PHYSIOLOGY OF THE ADRENOCORTICAL TISSUE IN THE WHITEFISH, COREGONUS LAVARETUS, LINNAEUS

Joan D. Fuller

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews

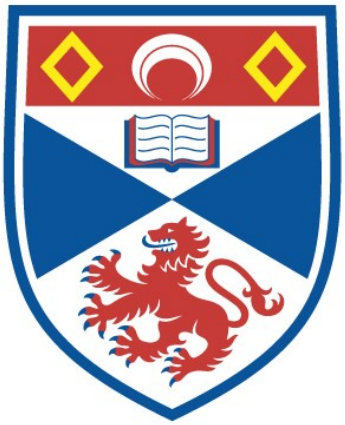

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PLATE 1. Frontispiece

1. Coregonus lavaretus (Linnaeus), the powan of Scotland; mature female, length 28 cm., August.

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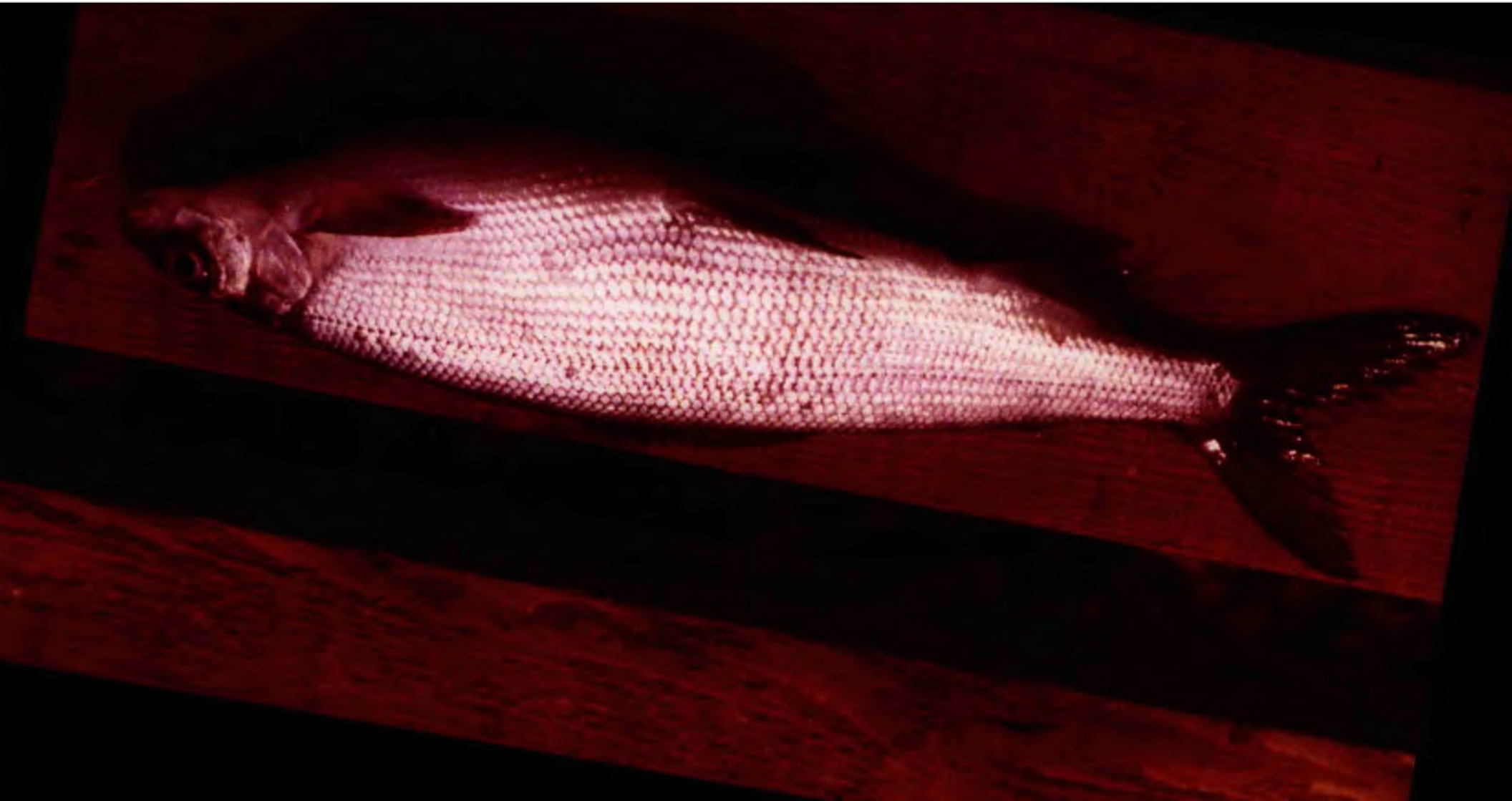

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Coregonus lavaretus (Linnaeus)

by

Joan D. Fuller

Department of Zoology

University of St. Andrews

A thesis submitted for the Degree of Doctor of Philosophy

November, 1974

CERTIFICATE

I certify that Miss Joan D. Fuller has spent 12 terms at research work on adrenocortical physiology of the whitefish, Coregonus lavaretus (Linnaeus), that she has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that she is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

D.B.C. SCOTT

(Supervisor)

November 25th, 1974.

DECLARATION

I declare that this thesis is the result of my own work. Where observations and experiments performed by others are referred to in the text, they have been acknowledged. Some of the work described in Chapters 3, 4 and 5 is being published in association with R. Fraser and D.B.C. Scott in the Journal of Endocrinology (in press). None of the material in this thesis has been submitted by me for any other degree.

J.D. FULLER

(candidate)

November 25th, 1974.

UNIVERSITY CAREER

I began my University career in St. Andrews in October, 1967. In June, 1971, I graduated with a 1st class Honours B.Sc. in Zoology and was awarded the Darcy Thompson Medal. My Honours B.Sc. thesis was a study of the reproductive biology of the Malaysian Osteoglossid teleost, *Scleropages formosus*, Müller and Schlegel, and the morphology of its pituitary gland (Fuller and Scott, J. Fish. Biol. in press). The material for this thesis I collected when I took part in an expedition to Tasek Bera, Malaysia in 1971, led by my Supervisor D.B.C. Scott and financed by a Government Grant-in-Aid. In 1972 I was awarded a Winston Churchill Fellowship and a National Geographic Society Grant to travel to the Amazon to make a comparative study of the S. American Osteoglossid, *Osteoglossum bicirrhosum*, Vandelli, with that of the Malaysian Osteoglossid.

In October, 1971, I began research into the structure and function of the adrenocortical tissue in teleosts with reference to reproductive physiology in *Coregonus lavaretus* (Linnaeus), the whitefish, and to social behaviour in *Xiphophorus helleri*, Heckel, the swordtail. This thesis presented for a Ph.D. represents the study of adrenocortical physiology in *Coregonus lavaretus*.

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Chapter 4 cont:

Chapter 5 cont:

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Steroid nomenclature and abbreviations used in text.

Trivial name	Systematic name
aldosterone	11β,21-dihydroxy-4-pregnene-3,20-dione-18-al
cortisol	11β,17α,21-trihydroxy-4-pregnene-3,20-dione
11-deoxycortisol	17α,21-dihydroxy-4-pregnene-3,20-dione
cortisone	17α,21-dihydroxy-4-pregnene-3,11,20-trione
20-dihydrocortisone	17α,20,21-trihydroxy-4-pregnene-3,11-dione
11-deoxycorticosterone(DOC)	21-hydroxy-4-pregnene-3,20-dione
18-hydroxydeoxycorticosterone (18 hydroxy DOC)	18,21-dihydroxy-4-pregnene-3,20-dione
progesterone	4-pregnene-3,20-dione
17α-hydroxyprogesterone	17α-hydroxy-4-pregnene-3,20-dione
17α-hydroxy-20-dihydroprogesterone	17α,20-dihydroxy-4-pregnene-3,20-dione
pregnenolone	3β-hydroxy-5-pregnene-20-one
corticosterone	11β-21-dihydroxypregn -4-ene-18-ol-3,20-dione

CPB - competitive protein binding

GLC - gas liquid chromatography

CBG - corticosteroid binding globulin

17 OHCS - total 17,21-dihydroxy-20-ketosteroids

cpm - counts per minute

dpm - disintegrations per minute

rpm - revolutions per minute

S.D. - standard deviation

μg - 10^{-6} gram.

ng - 10^{-9} gram.

pg - 10^{-12} gram.

LH - luteinising hormone

ACTH - adrenocorticotropic hormone

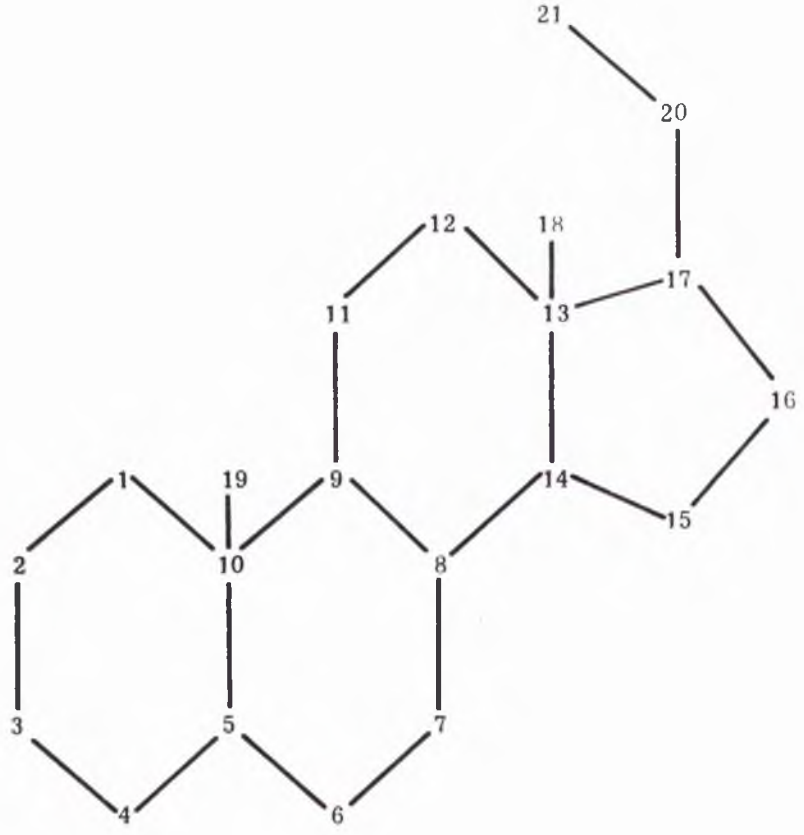


Fig.1. The pregnane nucleus, basic structure of adreno-corticosteroid molecules.

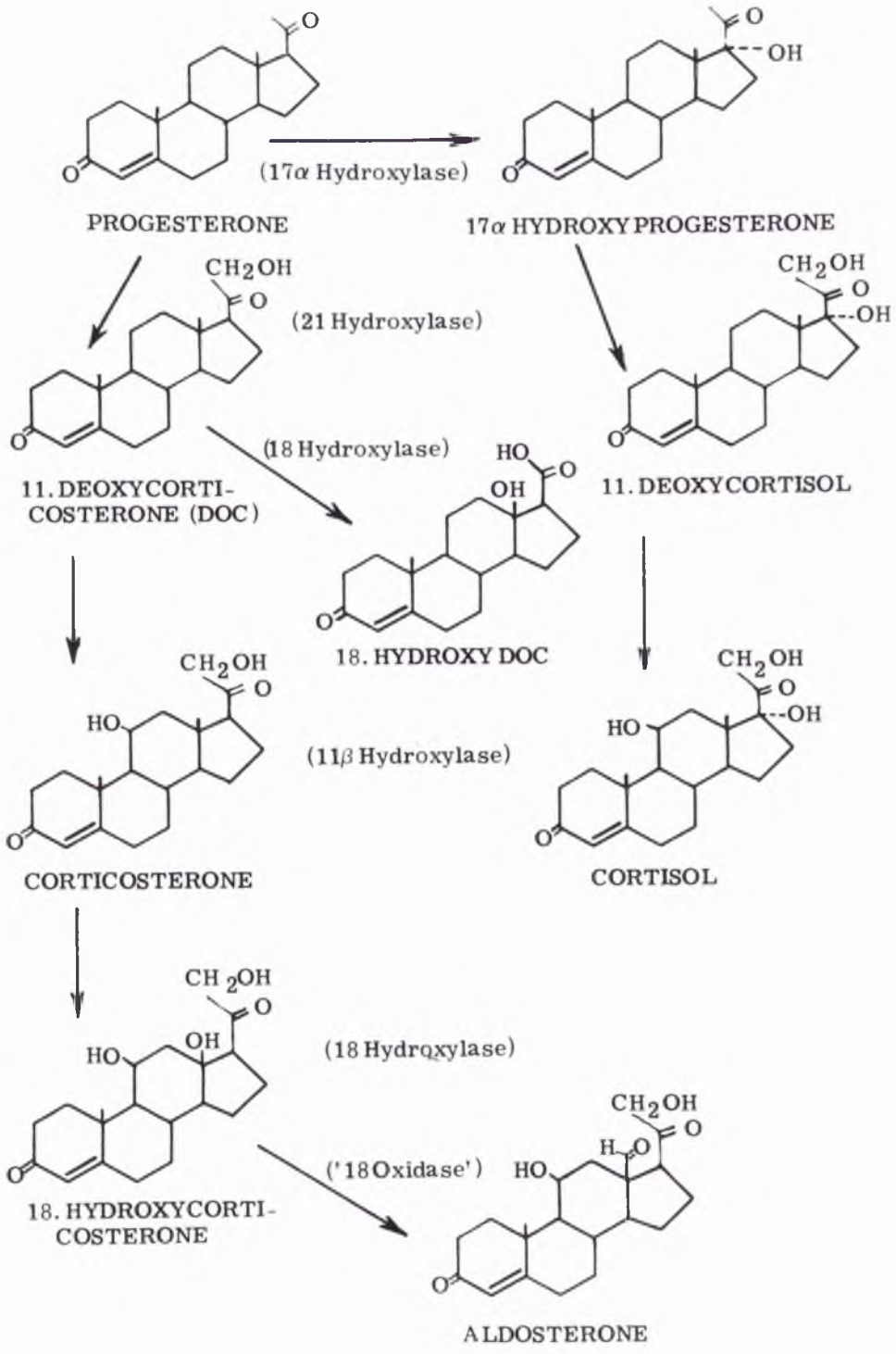


Fig. 2. Biosynthesis of corticosteroids.

GENERAL INTRODUCTION

The study of the endocrinology of the non-mammalian vertebrates has, in general, lagged behind the study of mammalian endocrinology, because the clinical importance of understanding the latter gave an early impetus to mammalian studies. More recently, partly because of their inherent evolutionary significance, but largely because of their importance as a source of protein for human consumption, attention has been focussed on the endocrinology of the bony fish (Osteichthyes). The teleost fish, partly because they comprise most of the commercially important groups, and almost all species used in fish-culture, have received particular attention (Bardach, Ryther and McLarney, 1972). Manipulation of the physiology of species cultured under artificial conditions is clearly of the greatest importance in fish-culture.

Of particular relevance at the present time are three physiological processes; all of which, it has been postulated, are under endocrine control. They are:-

(a) Osmoregulation. Several commercially important species (e.g. the salmon species; Chanos, and Hilsa) are anadromous, or at least migrate to regions of differing salinities at different seasons. This can be inconvenient in fish farming, as indeed can the requirements of stenohaline species.

(b) Stunting. Most species of teleosts kept under crowded conditions fail to grow, though they may mature and reproduce at a small size. This phenomenon has been linked by some authors with the concept of 'social stress' (Barnett, 1964). In practical fish-culture, the phenomenon is generally avoided by stocking ponds in such a way as to avoid reproduction, so avoiding overcrowding.

(c) Reproduction. Some species fail to reproduce under abnormal environmental conditions as the environmental clues which trigger hypothalamic-pituitary-gonad activity are absent. A few species can be induced to spawn by injections of pituitary extracts, but with varying degrees of success (Hardach, Hyther and McLarney, 1972). Failure of the reproductive cycle is usually at the stage of ovulation.

There is some indication that, in teleosts, all three processes are mediated to some degree by the adrenocortical hormones (Chester Jones et al., 1969). Osmoregulation and ionic balance have been attributed to pituitary prolactin, 8-arginine oxytocin and to adrenocortical hormones. The concept of 'social stress' involves adrenocortical activity stimulated by pituitary adrenocorticotropin. Reproduction is largely under the control of pituitary gonadotropins, but there is some evidence, albeit circumstantial and unsubstantiated, that adrenocortical hormones may be involved at the critical stage of ovulation. In addition, the adrenocortical hormones are probably involved in protein and carbohydrate metabolism and resistance to stress. The adrenocortical hormones are steroids (Fig. 1) and the biochemical pathways of the main adrenocorticosteroids are described in Fig. 2.

However, much of our present knowledge of the role of adrenocortical hormones is based on extrapolation of information gleaned from mammalian studies, and such extrapolations have proved wrong in the past (e.g. the role of prolactin in teleosts). Results derived directly from studies on teleosts have appeared during the past 15-20 years, but they have been, for the most part, isolated studies on specific topics, using random and often only remotely-related species. Adrenocortical studies have been made on at most 0.001% of extant teleost species, and it is not possible to give a general synthesis.

The aim of the present work is to investigate the physiology of the adrenocortical tissue of a single teleost species, to discover (a) the structure of the adrenocortical tissues and their relationship with other organs (b) the hormones secreted by the adrenocortical tissue, (c) the concentration of certain of these hormones, and how they are affected by the necessary processes of catching and handling the fish, (d) whether there is any correlation between adrenocortical activity and the annual reproductive cycle, and (e) whether there is any evidence for a pituitary-adrenocortical axis controlling ovulation.

In chapter 1, an account is given of the general and reproductive biology of the species chosen for study, Coregonus lavaretus (Linnaeus), the powan of Loch Lomond, Scotland. Amongst the 20,000 - 40,000 species of the polyphyletic teleost group, there are clearly likely to be differences in adrenocortical anatomy and physiology. Consequently, it seems most profitable to concentrate on a single species, and in particular a species whose general and reproductive biology can be studied in detail to provide a basis for these investigations. Moreover, the species should be such that variables such as catching, maintenance and killing techniques, which might affect results, can be closely monitored. The species, Coregonus lavaretus, fulfils these requirements.

In chapter 2, the anatomy and histology of the adrenocortical tissue and associated organs are described. Particular attention has been paid to the vascular and nervous supplies, and to identification of the chromaffin tissue, which is closely associated with adrenocortical tissue. For comparison, a number of other related salmonid species are also described.

In chapter 3, the identity of the hormones secreted in the plasma of the powan, and other salmonids, was investigated, using gas-liquid

chromatography. This provides not only an indication of which hormones are present, but also an indication of the concentrations at which they occur.

In chapter 4, two of the plasma corticosteroids identified, cortisol and 11-deoxycorticosterone were selected for more detailed study. Cortisol was chosen because it is present in highest concentration and 11-deoxycorticosterone because (although a mineralocorticoid in mammals) in teleosts it has been linked with ovulation, under pituitary luteinising hormone control. Saturation analysis was selected as the most suitable technique for the estimation of these steroids. The techniques and their validation are described in this chapter.

In chapter 5, the results of the plasma levels of cortisol and 11-deoxycorticosterone, measured by the saturation analysis techniques, are described. A study has been made to determine the effects of different catching and killing techniques, and the effect of aquarium maintenance on the steroid levels. Seasonal variations in plasma levels were correlated with the annual cycle, particularly the reproductive cycle.

Finally, in chapter 6, since adrenocortical activity is mediated by the pituitary, the relationship between them was investigated on a histological basis. This involved a study of the anatomy and histology of the pituitary gland of the powan. Also, because of the postulated role of corticosteroids in reproduction, *in vitro* studies of the effect of corticosteroid hormones on ovarian maturation were carried out.

CHAPTER 1.

SPECIES STUDIED

A. INTRODUCTION

Teleosts are a polyphyletic assemblage. Four main evolutionary divisions arose from holostean ancestors (Fig. 3), (i) the Clupeomorpha, (ii) the Elopomorpha, (iii) the Osteoglossomorpha and (iv) the Protacanthopterygii. The Protacanthopterygii form the main stem from which the modern teleosts have evolved; these are the Ostariophysi, the Atheriniformes, the Paracanthopterygii and the Acanthopterygii (Greenwood et al., 1966). The Salmoniformes are a relatively primitive protacanthopterygian order of about 37 families, of which the Salmonidae is one. All the species used in this present study are salmonids. The salmonids are distributed circumboreally in both marine and freshwater habitats. Some are purely marine (e.g. Osmerinae), many are anadromous (e.g. Salmoninae) and others are restricted to freshwater (e.g. Thymallus). The salmonids Coregoninae have a relict distribution in Britain; at the northern limits of their range (e.g. in the Baltic) they are anadromous, migrating from the sea to spawn in freshwater. In Britain, ancestral populations in the Celtic Sea may have behaved in this way, but changes in drainage patterns, and warming of the post-glacial seas, have resulted in the establishment of isolated, landlocked populations in cold, deep lochs in western Scotland, north-western England, Wales and Ireland (Maitland, 1970). The largest surviving Scottish populations are in Loch Lomond (commercially exploited until the beginning of the twentieth century) and in nearby Loch Eck. Smaller populations may still exist in Lochmaben, Dumfriesshire (Maitland, 1970).

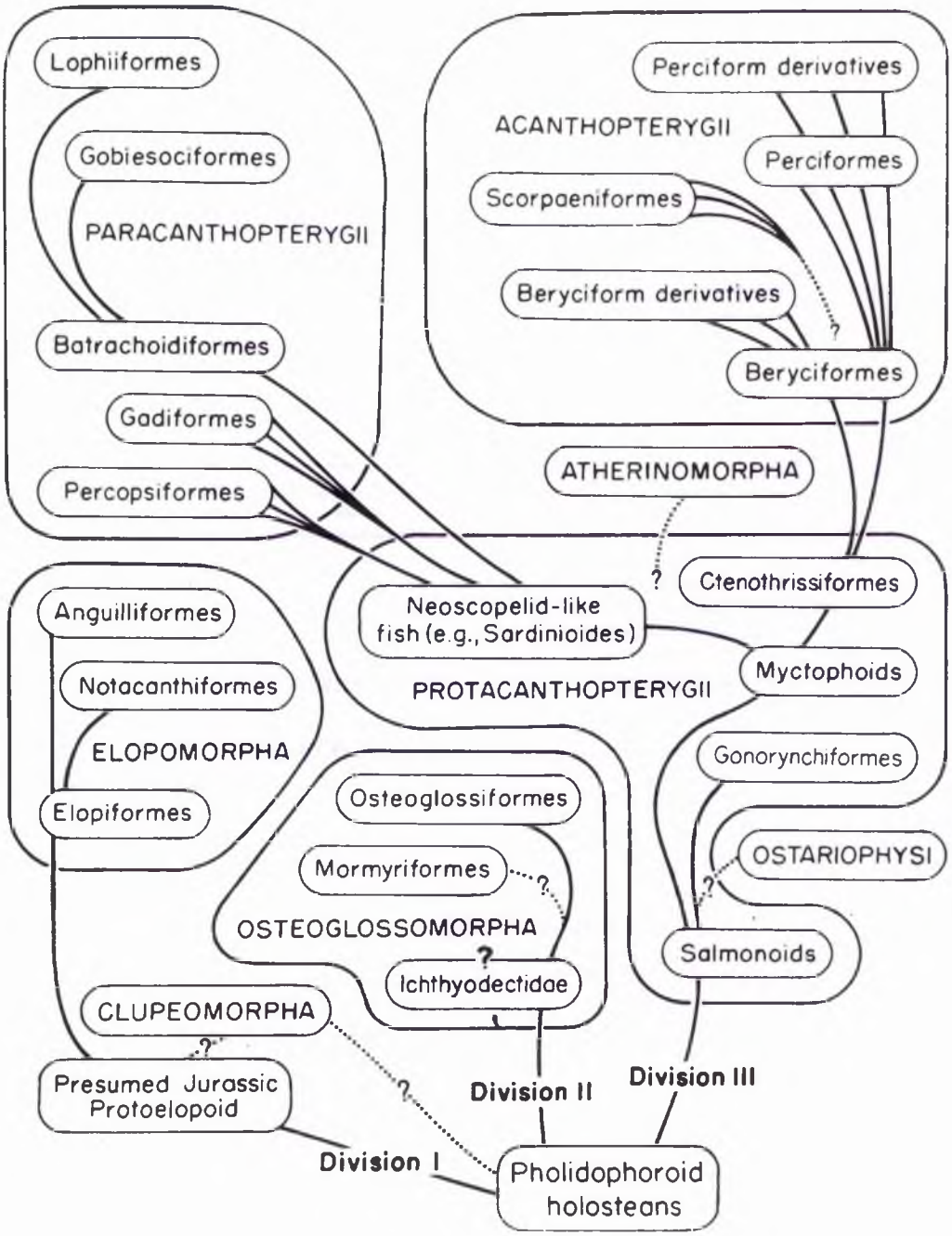


Fig.3. Phylogeny of the teleosts.

The taxonomy of the many endemic populations of Coregonus in Europe is uncertain. The population in Loch Lomond, on which the present study was carried out, is Coregonus lavaretus (Linnaeus) or Coregonus clupeoides (Lacépède) locally called powan (Maitland, 1970).

Coregonus lavaretus was chosen as the main species for this investigation of adrenocortical physiology for practical reasons. It is abundant in Loch Lomond, where it is probably the commonest species (Slack, Gervers, Hamilton, 1957). It is feasible for the research worker to net the fish himself, so that the exact conditions to which they have been exposed before analysis can be rigorously controlled. The proximity of the University of Glasgow Field Station ensures rapid processing of the fish with minimum delay after catching. Reasonable sampling of fish can be obtained throughout the year (but see p. 12). The general biology of the species has been studied (Slack, Gervers, Hamilton, 1957) and in particular the reproductive cycle is known (Maitland, 1968) though no histological study of the gonadal cycle has been made. The spawning period is clearly delimited; it occurs during the first 3 weeks of January, and spawning takes place on off-shore gravel banks; some of which have been positively identified (Fig. 4). During the spawning period it is possible to identify with accuracy the exact physiological state of the female, whether ripe but pre-ovulating, actively ovulating or spent, for correlation with hormone levels. Though it is a relatively small species, only exceptionally as much as 37 cm. in total length, and consequently easy to handle, as much as 2.5 ml. of blood can be taken from a single specimen for hormone analysis. The only real disadvantage of the species is its intolerance of confinement, specimens seldom survive unless rapidly transferred from shore seine-nets to very large, preferably circular, aquaria.

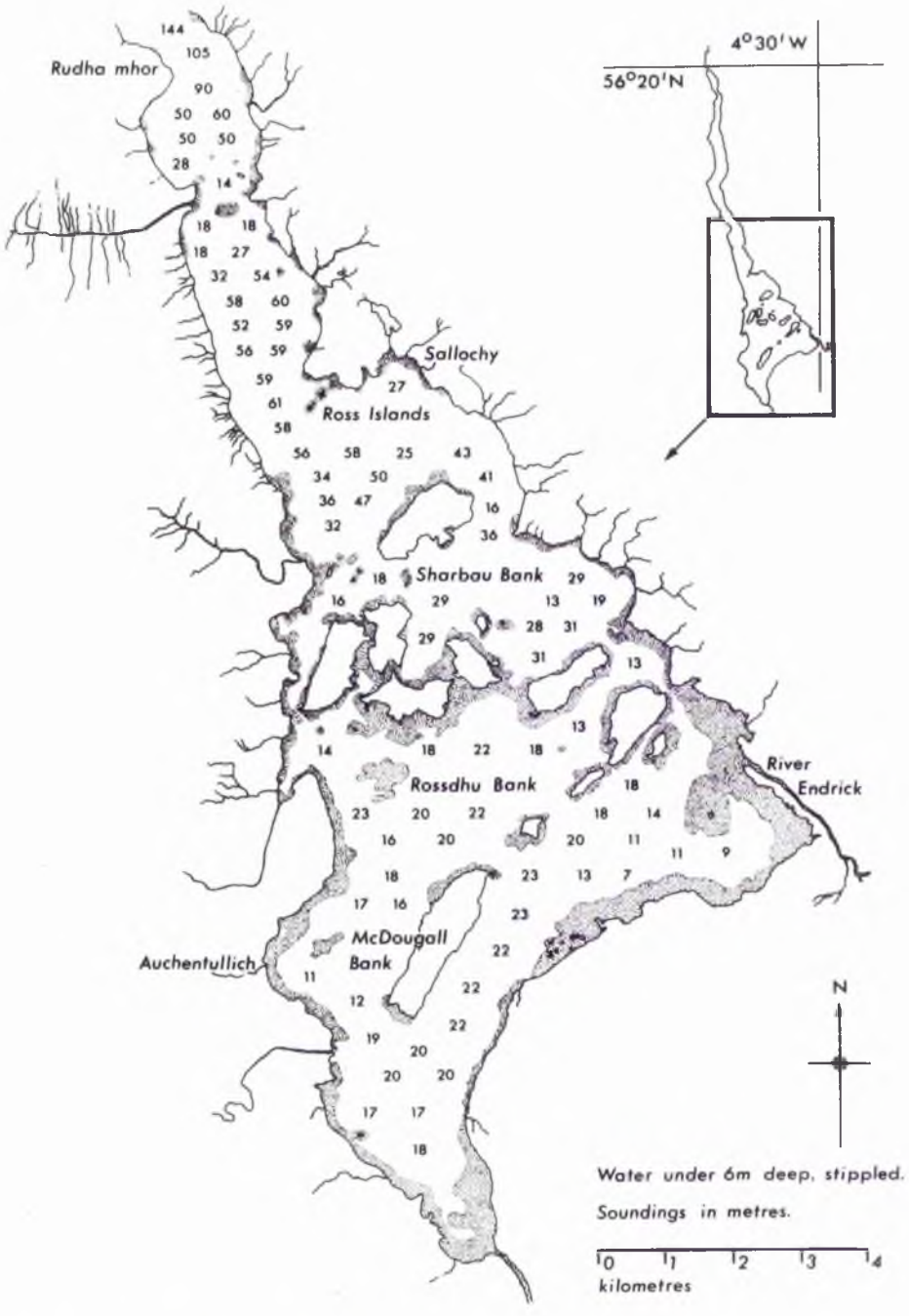


Fig.4. Southern region of Loch Lomond

As with any other teleost used for experiment, obtaining specimens involves some more or less drastic catching technique, some degree of handling, perhaps maintenance in aquaria for varying periods before use, and a variety of killing methods. Each of these factors is potentially capable of inducing change in endocrine activity, and their effects must be assessed. A range of such techniques has been used in the present study to provide comparative data on their effects on corticosteroid activity.

In addition to Coregonus lavaretus, the following species of Salmonidae have been studied, though in less detail:

from Loch Lomond,

Salmo salar,	Atlantic salmon	(anadromous)
Salmo trutta trutta,	sea trout	(anadromous)
Salmo trutta fario,	brown trout	(fresh water)

from other localities,

Salmo gairdnerii,	rainbow trout	(fresh water, hatchery stock)
Salvelinus willughbii,	char	(fresh water, Windermere)
Osmerus eperlanus,	smelt	(marine, Plymouth).

Aim of this section of the project.

The aim of the work described in this chapter is to clarify the general biology of the species, Coregonus lavaretus, the powan, in particular its reproductive biology, as a basis for the endocrine studies described in ensuing chapters.

2. MATERIALS AND METHODS

1. Collecting methods.

Specimens were caught by two methods:

(a) Benthic gill-netting. Gill nets of No. 0 nylon thread and of 3.5 to 5 cm. stretched mesh (Norsenet, Bergen) were set throughout the year on the lake bottom in the region of the field station, between 1600 and 2000 hrs, and were lifted between 1000 and 1400 hrs. the following day, to avoid possible diel variations in steroid levels. The nets were set between 2 and 30 m. deep, depending on the season. During January, in addition, nets were set on the spawning ground, between and in the immediate vicinity of the Rose Islands (Fig. 4). Using this catching method fish are enmeshed in the net for up to eighteen hours (except in experiments to determine the effect on steroid levels of length of time spent in the net, P. 89). As the fish were removed from the net they were placed in tubs of loch water and transported to the laboratory. They were killed either by cerebral concussion, or by immersion in a lethally concentrated solution of MS222 (Sandos).

(b) Shore seine-netting. A seine-net 45 m. long, 2.5 m. deep and of 5.0 cm. stretched mesh was set at dusk, about 75 m. off shore in Sallochy Bay (fig. 4), and hauled onto the beach. Using this method the fish are not enmeshed and remain in the net for only about 15 mins. Divers observing a haul reported that the fish were undisturbed by the net until the last 2 or 3 minutes. As soon as the net was brought ashore the fish were removed and killed either by cerebral concussion or MS222 anaesthesia. Unsuccessful attempts were made to seine-net on the Rose Island spawning ground during the spawning season in January. Attempts at another known spawning ground, the McDougal Bank (Fig. 4) were also unsuccessful.

(c) Electrofishing has been proposed as a stress-free catching technique (Scott, 1963), but unfortunately permission to electrofish on Loch Lomond was not available.

(d) Maintenance of specimens in aquaria for varying periods before use is normal practice. To establish the effect of aquarium maintenance, specimens were kept in polythene aquaria for up to 80 hours. They were not fed during this period and the effect of longer-term aquarium maintenance was not investigated.

2. Blood sampling.

Blood was collected within one hour of killing the fish, except in experiments to determine the effect of delay (p. 88). The fish were opened mid-ventrally, from the cloaca to the gills, exposing the heart. A disposable glass pipette was inserted through the ventricle (which acted as a seal, preventing escape of the blood or its contamination by body fluids) through the atrium and sinus venosus into the right duct of Cuvier, from which the blood was withdrawn. Gentle pressure along the posterior cardinal veins at the same time drove more blood through the pronephros (where the adrenocortical tissue is situated) to the duct. The detailed anatomy of this region is described later (p. 25). On average 1 ml. of blood could be collected from each fish in this way, though occasionally as much as 2.5 ml. was obtained. Separate pipettes were used for fish of each sex and of different reproductive stages. All pipettes, were rinsed, between specimens, in dilute heparin solution. Samples were kept on ice during collection. Each sample was centrifuged at 4500 rpm for 15 minutes, and haematocrits were taken. (As the hormone measurements to be described (Chapters 3, 4 and 5) are expressed in terms of hormone/100 ml. of plasma, it is obviously necessary to know whether the ratio of plasma to whole

blood is variable, as any future results may be from measurements on whole blood). The plasma was pipetted off into glass vials, frozen in solid CO_2, and stored at -20°C. Samples from individual fish were kept separate unless otherwise stated.

The further processing of these blood samples for identification and measurement of plasma corticosteroids is described later (pp. 36, 52, 64).

3. Histological technique.

Gonads for histological examination were dissected from fish immediately after death and as rapidly as possible. Routine fixation was in Bouin's aqueous fixative (Pantin, 1946) for 24 hours, followed by dehydration in 30%, 50% and 70% ethanol. Picric acid was washed out by repeated changes of 70% ethanol. The tissue was then further dehydrated in a 2-methylpropan-2-ol (TBA) series:

70% embedding alcohol	(aq. dest., TBA, 95% ethanol, 3:2:5)	2 - 4 hrs.
85% " "	(" " " " " , 3:7:10)	2 - 4 hrs.
95% " "	(" " " " , 1:9)	2 - 4 hrs.
100% " "	(TBA, absolute ethanol, 1:1)	2 - 4 hrs.
TBA	3 changes, 2, 16 and	2 hrs.
TBA, paraffin, 1:1		6 - 12 hrs
"fibrowax" (Arnold B. Horwell, Ltd.)	3 changes, 2, 16 and	2 hrs.

Sections were cut at 5 µm on a Leitz rotary microtome. Gonad sections were routinely stained in lead haematoxylin (MacConnail, 1947) for 2½ hrs. and counterstained in erythrosin, 1% aqueous for 1 min. The histological techniques used for other tissues are described where relevant in the text (pronephros p. 19 ; embryos p. 19 ; pituitary gland p.103-4).

4. Assessment of reproductive state.

The reproductive state of the fish was determined by:

(a) The gonadosomatic ratio - ratio of gonad weight to body weight (excluding gonad) - expressed as a percentage. Not less than 100 fish were collected by gill-netting each month throughout the year. The mean monthly gonadosomatic ratio was calculated for males and females separately.

(b) Histological examination of the gonad. Transverse sections through the middle region of the ovary and testis of 3 fish of each sex, whose gonadosomatic ratios were close to the monthly mean, were examined. In males, the proportion of early spermatocyte stages to spermatozoa was subjectively compared. In females, the proportions of primary and secondary oocytes, ova and atretic oocytes were assessed (Scott, D.B.C., 1974).

(c) Visual inspection of the ovary. During the spawning period in January, it proved possible to assess whether individual females (i) had not yet begun to ovulate, (ii) were on the point of ovulating, (iii) were actively ovulating or (iv) were spent.

The somatic condition factor - ratio of body weight (excluding gonad) to total length^3 - was also measured for each of the 100 fish in the monthly samples. This parameter gives an indication of the general fitness of the fish, in terms of food reserves (Deason and Hile, 1944), and in some species, at least, shows an annual cycle associated with the reproductive cycle (Scott, D.B.C., 1963, 1974). The condition factor, calculated on the basis of total weight (including gonad) was used in a previous study of powan biology (Maitland, 1968).

5. Maintenance of embryos.

Ova from ovulating gill-netted females were stripped into a dry vacuum flask and milt from several ripe males was added. After 5 mins. the fertilised ova were washed in several changes of water. The ova were maintained in plastic trays with a constant flow of tap-water at 5 - 8°C until hatching 2½ months later. The trays were inspected daily, dead and infected ova were removed. Mortality is heavy during the first few days and again at hatching, the latter probably because of the combination of flowing water and lack of a suitable substrate. On hatching, the fry were transferred to polythene tubs 1 m. in diameter, without running water, and were fed on freshwater zooplankton. Fry from hatching to 9 months were obtained in this way for anatomical study (p. 19). In addition, a number of specimens were kindly provided by Dr. P.S. Maitland, (Nature Conservancy, Edinburgh), from embryos collected the previous year, and reared in a similar way (Maitland, 1967).

C. RESULTS AND DISCUSSIONS

1. General biology.

About 800 specimens of powan were caught by seine-netting during the present study. Seine-netting was unproductive between November and April, presumably because the fish do not inhabit shallow inshore waters at this time (Nikolsky, 1963). This was particularly regrettable, as the availability of ripe and spawning fish caught by seine-netting rather than by gill-netting would be very valuable for hormone analysis (p. 83). Consequently, considerable efforts were made to seine-net on the spawning grounds themselves, in January. The nature of the lake-bottom, however, in combination with winter gales and blizzards defeated these efforts in two successive years and fish between November and April were caught by gill-netting only.

Contrary to previous reports (Maitland, 1968), seine-netting was also unproductive from late June to late August. This may be due to the recent influx of tourists in the region of Sallochy Bay or to other ecological changes. Whatever the cause, the result was that relatively few specimens of seine-netted fish were obtained.

Over 2500 specimens were caught by gill-netting. As in the case of seine-netting, fish were scarce from July to September. At this time of year coregonids are believed to migrate from deep water during daylight to inshore surface waters at night. It may be that the factors responsible for keeping the powan away from inshore waters in the hours of darkness (observed in this study) result in the fish inhabiting offshore surface waters at this time, in which, case, pelagic gill-nets set overnight might have caught powan from July to September. Unfortunately such nets would have been exposed to damage by tourists, and so could not be used.

The fish collected ranged from 10 to 37 cm. in total length. During most of the year the sex ratio was approximately 1:1, though individual samples often departed markedly from this ratio, suggesting that the fish may form more or less unisexual shoals. Diseased fish were common during the months following spawning, being infected mainly with Saprolegnia. During the rest of the year about 1 fish in 50 was infected. A few gill-netted fish were damaged, either by cormorants or lampreys, or by net damage in rough weather. All such damaged and diseased fish were discarded, or separately analysed (p. 91).

2. Reproductive biology.

(a) Males mature in their second year. The testes are paired, and when immature (March - May) consist of seminiferous tubules lines with spermatogonia, with interstitial cells between the tubules. Between June and

September later stages of spermatogenesis occur in cysts in the tubule walls, so that in section each tubule consists of a number of juxtaposed cysts at differing stages of development (Plate 2a). Spermatids and spermatozoa appear in the lumen of the tubules towards the end of this period. From October to January the tubules are packed with spermatozoa, and the tubule walls are stretched and thin (Plate 2b), with very few earlier spermatocyte stages remaining.

During the first 2 - 3 weeks of January, ripe males migrate to the spawning grounds where they remain until their spawning is over. As the spermatozoa are shed, the testis becomes highly vascularised, and the tubule walls thicken. Initially sperm remain in the tubules (Plate 2c), but by February must have been resorbed and the tubules become occluded and contain fatty deposits (Plate 2d).

The mean gonadosomatic ratio of males (Fig. 5) is at its maximum of about 2% of body weight from September to January, with no significant increase during these months, and falls gradually thereafter to a minimum of 0.4% in June or July. Rapid recrudescence takes place between July and September.

(b) Females also mature in their second year. The ovaries are paired (Plate 4a), and in immature fish contain only primary (pre-vitellogenic) oocytes (Plate 3a). Maturation begins in May with the development of secondary oocytes (with yolk precursors, and chorion), (Plate 3b). From July until December there is a progressive increase in the number of ripe ova and the number of secondary oocytes decreases to zero. Ripe ovaries thus contain only primary oocytes and ova (Plate 3c).

Before and during the spawning period in January, the ripe females cruise in unisexual shoals near the spawning grounds. As each individual

fish ovulates, it migrates to the spawning grounds, spawns, and then returns to the open water. Consequently, gill-netting on the spawning grounds produces a sex ratio of about 10♂ : 1♀. The ovaries of ripe, preovulating females are compact and the oocytes are opaque. The first sign of ovulation (called "barely ovulating" in the remainder of this study) is a reduction in the compactness of the ovary and the oocytes become translucent. Actively ovulating fish have translucent ova released from the follicles and escaping from the cloaca. After spawning, the ovary contains only primary oocytes, follicular calyces, and a few unspawned ova which undergo atretic resorption (Plate 3d).

The gonadosomatic ratio of females (fig. 5) rises progressively from July to December, 16%, unlike the males' sudden increase between July - September. After spawning, the female gonadosomatic ratio falls suddenly and remains low, about 1%, for 6 months, until July.

(c) The somatic condition factors (fig. 5) of both sexes increase from their minimum values in July to their maximum in September, at much the same time of year as gonad recrudescence occurs. Values fall slightly during the winter, prior to spawning, and then more steeply after spawning, especially in the females. These observations accord reasonably well with somatic condition factor changes in other species (Scott, 1963). However, minimum levels in the present study were found in June or July in both sexes whereas Maitland (1968) observed minimum condition factors in powan soon after spawning, these results were based on only 10 - 30 fish/month in 1964-6, but he used condition factor (not somatic condition factor) which includes gonad weight. The present study indicates that after spawning there is a period of 5 months in which somatic condition factors continue to fall, unlike other species in which they rise soon after spawning (Scott, 1974).

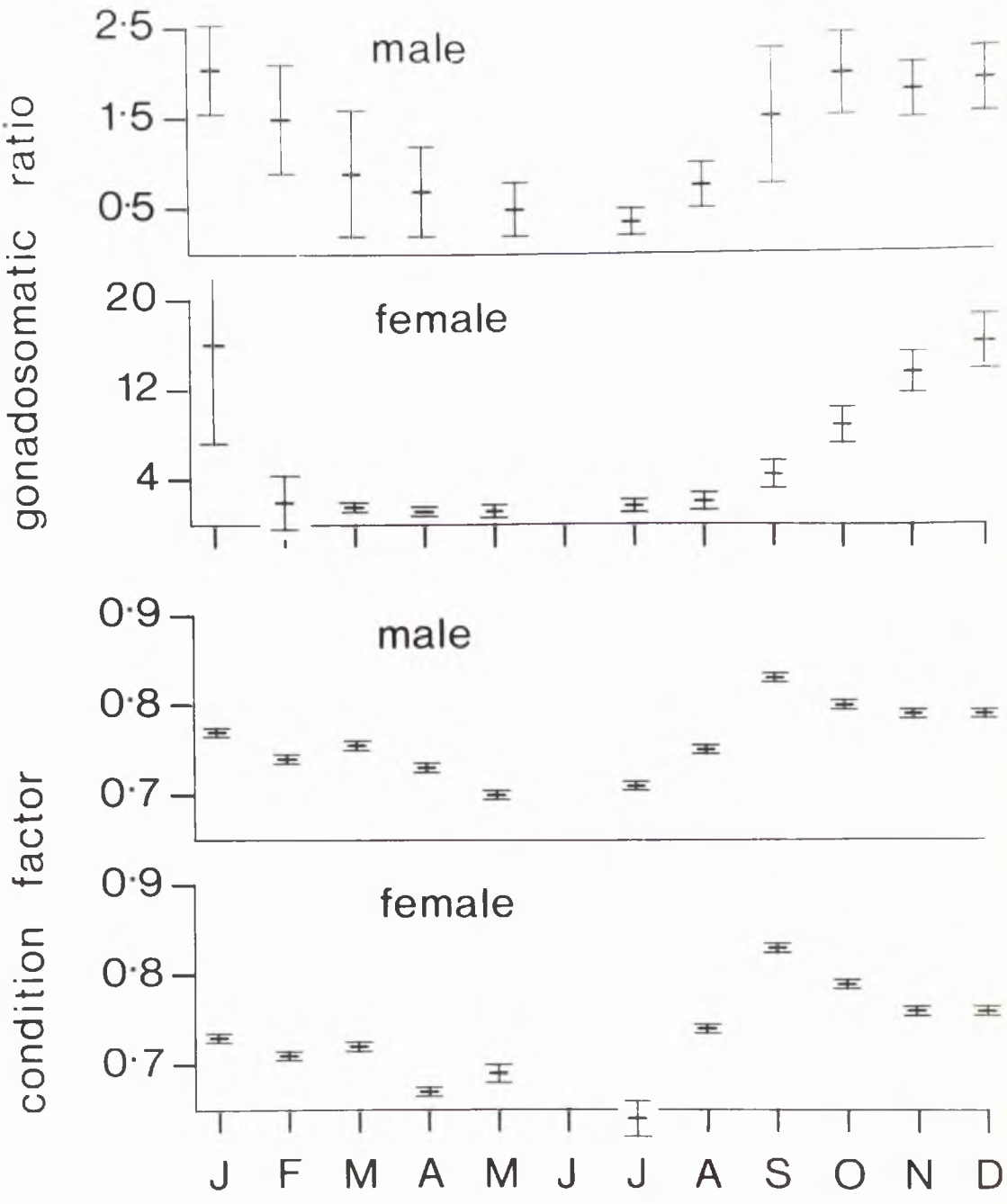


Fig. 5. Gonadosomatic ratios and somatic condition factors of Coregonus lavaretus, (mean ± S.D.).

(d) Haematocrit values are listed here mainly as reference for future research work on steroid levels in the powan. Recent research is being carried out on steroid levels in whole blood of mammals (Fraser, pers. comm.) for comparison with plasma steroid levels. It may therefore be of importance to note the variation in the ratio of plasma to whole blood volume in the species whose plasma steroid levels are described in this study (Chapters 3, 5).

Haematocrit values were taken from 95 samples of individual powan blood. Values were expressed as the percentage volume of red blood cells in whole blood (Table 1a). In general, haematocrits are significantly higher in males than females (Table 1b).

TABLE 1a: Haematocrits of Coracanus lavaretus, expressed as percentage red blood cell volume in total blood volume, ± 1 standard deviation.

Month	Sex	Reproductive state	No. of samples	Haematocrit
(a) January (spawning-time)	♂	ripe	29	51.3 ± 11.7
	♀	pre-ovulating	2	40.2 ± 0
		barely ovulating	2	42.5 ± 0
		ovulating	15	36.8 ± 6.6
(b) February	♂	spent	14	46.6 ± 5.2
	♀	spent	12	35.7 ± 6.0
	♂/♀ pool	spent	6	42.6 ± 5.5
(c) September	♂	mature	5	50.8 ± 6.3
	♀	mature	10	44.0 ± 5.7
(d) Aquarium maintained (January)	♂	ripe	8	46.8 ± 8.5
	♀	pre-ovulating	1	38.9
	♀	ovulating	1	31.0

TABLE 1b: Statistical analysis of haematocrit variation, significance accepted at the 1% level.

Groups compared	P value
September ♂ v. September ♀	> 0.05
January ♂ v. January ♀ (ovulating)	< 0.001
February ♂ v. February ♀	< 0.001
September ♀ v. January ♀ (ovulating)	< 0.1
February ♀ v. January ♀ (ovulating)	> 0.05
February ♀ v. September ♀	< 0.1
Aquarium ♂ v. January ♂ (January)	> 0.05

CHAPTER 2.

THE ADRENOCORTICAL AND CHROMAFFIN TISSUES

A. INTRODUCTION

1. Adrenocortical tissue.

The adrenocortical tissue of vertebrates is derived, like the gonad, from coelomic mesoderm. Its location in the adult is different in the various vertebrate classes. It forms a discrete glandular mass (the "adrenal corter") in the Mammalia and Aves, but is generally more dispersed in the other vertebrates, though always associated with the kidneys (Chester Jones, 1957). In teleosts a range of anatomical types exists (Nandi, 1962). Typically the adrenocortical (= interrenal) tissue is associated with the posterior cardinal veins or their tributaries, close to their junction with the anterior cardinal veins and the ducts of Cuvier. The adrenocortical hormones are therefore secreted into blood derived mainly from the kidneys.

The functional kidney of adult teleosts is the mesonephros (strictly called the opisthonephros, as it contains both meso- and metanephric components), which extends along most of the length of the coelom. In the embryo, and for a short time after hatching, the functional kidney is the pronephros, but in the adult it is either transformed into a lymphoid organ (the "head kidney") or it disappears. The pronephros of the adult may be a discrete organ, separate from the mesonephros (e.g. Phoxinus (Scott 1963); Perca (Weatherley 1963); the Osteoglossidae (Fuller and Scott, unpublished)), or continuous with the mesonephros (e.g. most of the salmonids described in this work). The posterior cardinal veins pass through the pronephros, and the adrenocortical tissue occurs here, as single cells, groups of cells, or in layers round them and their tributaries (Nandi, 1962). In the aberrant Osteoglossidae the pronephric lobes are permeated by sinusoids derived from the posterior cardinal veins, and the adrenocortical tissue forms large

discrete islets among these sinusoids (Fuller and Scott, unpublished). In those species without a pronephros, the adrenocortical cells occur near the posterior cardinal veins amongst functional mesonephric glomeruli and tubules (e.g. Xiphophorus (Fuller, unpublished).

2. Chromaffin tissue.

Encapsulated within the adrenocortical tissue in birds and mammals is the main mass of the chromaffin tissue (the "adrenal medulla"). An association of these two tissues is also described in teleosts, where the chromaffin cells are more or less intermingled with the adrenocortical cells in the pronephros (Mandi, 1962). However, the identity of the presumed chromaffin tissue in teleosts has never been critically confirmed. Most identifications have been based on simple histological stains (Oguri, 1960). A few workers have used the classical chromaffin reaction in some form (Hillarp and Hökfelt, 1955; Banerji, 1973) but the response of teleost tissue to such fixation is very variable, and in any case the reactions involved are not histochemically specific. Other workers have used somewhat more critical histochemical techniques, but still with variable results, and still essentially non-specific (Tramezzani, Chiocchio and Wassermann, 1966).

In some species two different endocrine cell-types are not distinguishable in the pronephros, and as the single cell-type present reacts to histochemical stains for adrenocortical tissue (Scott, unpublished), chromaffin cells are presumably not present. Indeed, there is no reason why chromaffin and adrenocortical tissues should be associated, as their embryonic origins are quite different, the chromaffin tissue being derived from autonomic (sympathetic) ganglia; and their only physiological interaction is by hormonal pathways via the pituitary gland. Even in mammals, although the main mass of chromaffin tissue lies in the adrenal medulla.

many other chromaffin bodies occur, chiefly near the sympathetic ganglia. It would be reasonable to expect that wherever the chromaffin tissue is, it will be innervated by sympathetic nerves.

The identification of adrenocortical tissue itself is often uncritical, depending largely on observations that the presumptive adrenocortical cells react by histological changes to some form of stress (Oliveresu, 1965; Hanke and Chester Jones, 1966). Many other tissues such as thyroid and, obviously, chromaffin would also react under these conditions. In some species, histochemical identification has been carried out. In vitro studies (pp. 31-33) have necessarily been on incubates containing extraneous tissues, possibly including chromaffin.

The aim of this section of the project is to describe the anatomy and histology of the pronephros of Coregonus lavaretus, and related salmonids, with particular attention to its vascular supply and innervation; and to identify critically the adrenocortical and chromaffin tissues by histochemical and immunological methods.

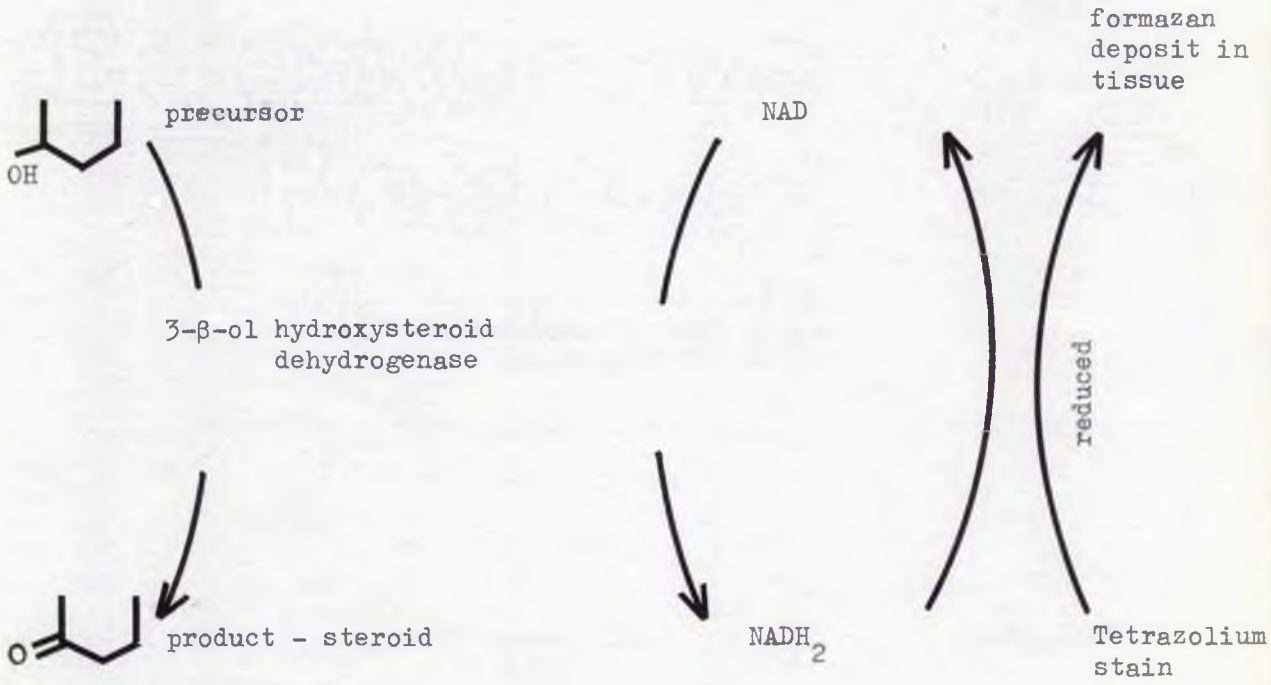
B. MATERIALS AND METHODS

1. Anatomy and histology.

Tissues were fixed, embedded and sectioned as described on p. 10 and stained in Masson's trichrome (Anon, 1969). Pronephroi of adult fish were serially sectioned horizontally at 5 µm. Entire larval powan, newly-hatched, and 1, 2, 3, 6 and 9 months old were serially sectioned sagitally at 5 µm. The anatomy of the pronephros and related organs was reconstructed by making serial isometric drawings of the sections (Figs. 6, 7).

2. Histochemistry.

(a) Adrenocortical tissue. Adrenocortical tissue was identified by a histochemical technique specific for 3-β-ol hydroxysteroid dehydrogenase (Wattenberg, 1959; Baillie, Ferguson and Hart, 1966; Simpson and Wardle, 1967). 3-β-ol hydroxysteroid dehydrogenase catalyses one reaction in the synthesis of adrenocorticosteroids occurring shortly after formation of the steroid nucleus (Fig. 1), the irreversible oxidation of precursors. This oxidation is nicotinamide adenine dinucleotide (NAD) mediated, and coupled to the reduction of a tetrazolium salt, it can localise the reaction in tissue sections by colour formation in the cells. In this method, frozen sections of adrenocortical tissue are provided with exogenous steroid precursor, NAD, and tetrazolium stain.



(i) Reagents:

Buffer, pH 7.6: 87% 0.2 M Na_2HPO_4 (14.4 g/200 ml. distilled water)

13% 0.2 M NaH_2PO_4 (3.1 g/200 ml. distilled water)

Reaction mixture: 70 ml NaCl 0.2 M (2.4 g/200 ml. distilled water)

30 ml Buffer

200 mg Nicotinamide (Sigma)

100 mg DPN (NAD, Sigma)

10 mg Nitro blue tetrazolium stain (Sigma).

Steroid substrate: 1 mg Dehydroepiandrosterone

OR

1 mg Pregnenolone

5 ml Methanol (BDH analar, redistilled)

0.5 ml aliquots of steroid substrate were transferred to separate tubes, evaporated under nitrogen and redissolved in 2 drops of diethyl foramide.

Substrate mixture: to each tube of steroid substrate were added 15 ml of reaction mixture. This substrate mixture was stored at -20°C.

Preserving solution: 10% formalin in NaCl (12.2 g/l distilled water).

(ii) Method. Pronephroi were dissected from freshly-killed maine-matted powan. They were snap-frozen on to cork-faced metal tissue-carriers by immersion in liquid nitrogen (-196°C), in an isopentane-liquid nitrogen mixture, or in liquid propane-propylene mixture, cooled by liquid nitrogen. Immersion in liquid nitrogen was the most convenient method, and as tissues

frozen in this way were in no way inferior to the other methods, this technique was used for all frozen tissues. Sections were cut at 12 µm on a Slee Cryostat at -11°C, flattened on to warm coverslips, and dried in a stream of cool air.

Of each group of 8 sections, 2 were fixed in preserving solution for 10 minutes, stained in Masson's trichrome, and mounted in Canada balsam, as reference sections. Of the remaining 6 sections, 2 were immersed in dehydroepiandrosterone substrate mixture for 20 minutes, 2 in pregnenolone substrate mixture, and 2 in control solution containing reaction mixture but no steroid substrate. One of each pair was first washed in cold acetone, the other transferred directly to the substrate mixture. All sections were then washed in distilled water for 10 minutes, in preserving solution for 10 minutes, again in distilled water for 10 minutes, and mounted in glycerine jelly. Staining intensity was not affected by the temperature of the substrate mixture.

(b) Chromaffin tissue. Several methods were used to identify chromaffin tissue. Only one proved satisfactory, and the others are therefore only described in outline below.

(i) Orth's fixative. A technique based on the classical chromaffin reaction (Baker, 1958).

(ii) Method of Hillarp and Hökfelt (1955). The method is based on the conversion of the oxidation products of adrenaline and noradrenaline to insoluble pigments by potassium dichromate at controlled pH. Tissues were treated in a solution of 5% potassium dichromate, 5% potassium chromate in water, pH 5 - 6, for 48 hours before fixation for 24 hours in 10% formalin. They were then snap-frozen in liquid nitrogen, reference sections stained in Masson's trichrome, and mounted in Canada balsam.

(iii) Oxidation with silver solution following glutaraldehyde fixation (Tramezzani, Chiocchio and Wasserman, 1966).

These authors identified adrenalin and noradrenalin cells in adrenal glands of man, other mammalian species, and the duck. Frozen sections were made of (a) unfixed glands, incubated in ammoniacal silver solution, (b) unfixed glands, washed in water, and incubated in silver solutions, and (c) sections fixed in 6.5% glutaraldehyde solution, washed in water and then incubated in silver solution. In (a) both adrenalin and noradrenalin are localised by reduced silver; in (b) both amines are washed out by the water, so that no silver deposition occurs; in (c) noradrenalin forms an insoluble compound with glutaraldehyde, and adrenalin is washed out, so that only noradrenalin-containing cells are localised.

(iv) Fluorescence histochemistry for the cellular demonstration of biogenic amines (Falck and Owman, 1965).

This method has been used to identify adrenergic nerves, and adrenalin and noradrenalin secreting tissues in vertebrates, including poikilotherms (McLean, Bell, and Burnstock, 1967; Read and Burnstock 1969; Cannon and Campbell, 1972). It has also been used to identify chromaffin cells in teleosts. The method is based on the principle that catecholamines can be converted to highly fluorescent trihydroxyindoles by exposure to formaldehyde vapour, providing that the reaction occurs in dry protein. Primary catecholamines can be induced to fluoresce by relatively short exposures; secondary catecholamines such as adrenalin require longer exposure.

Method. Pronephroi were dissected from fresh-killed seine-netted powan, and were cut in half sagitally. The right half was snap-frozen in liquid propane ("calor gas") cooled in liquid nitrogen. The tissue was stored in liquid nitrogen for 3 - 4 hours prior to being freeze-dried for 3 days at 10^{-6} mm Hg of vacuum. The tissue chamber was not cooled. The tissue was

then rapidly transferred with forceps to a beaker and placed in a 500 ml desiccator containing 5 g paraformaldehyde at 70% humidity. This was incubated at 80°C for 3 hours, and the tissue was then vacuum-embedded in paraffin wax. Sections were cut at 10 µm on a Leitz microtome within 24 hours, and mounted on dry microscope slides in one drop of liquid paraffin. The slide was examined on a Zeiss fluorescence microscope, in a warm air stream which melted the paraffin wax. Barrier filters 53/44 and exciter filters BG 12 (2) were used to observe green fluorescence in the 400 - 500 µm region. Alternate sections were stained in Masson's trichrome and mounted in Canada balsam as reference sections.

3. Immunostaining for adrenocortical tissue.

The fluroescent-antibody sandwich technique (Nairn, 1969) was applied to frozen sections of adult pronephroi prepared as described on p. 21 Rabbit antisera to aldosterone and to deoxycorticosterone were provided by the M.R.C. Blood Pressure Research Unit, Glasgow, and rabbit antiserum to cortisol by Dr. A.P. Dean. Fluorescent isothiocyanate conjugated anti-rabbit globulin was obtained from Burroughs-Wellcome Laboratories, and was used in a dilution of 1:8.

Unfixed 5 - 20 µm sections were air-dried on microscope slides for 15 minutes. They were then washed with two changes of either phosphate or tris buffer for 5 minutes each. Each section was then immersed in one of the 3 antisera for 20 minutes. A range of dilutions of antisera was used; incubation ranged from 30 minutes to 6 hours; and temperature was varied from 4°C to 40°C. Unbound antibody was washed off with buffer for 10 minutes, and the section was then immersed in labelled anti-rabbit Y globulin for 20 minutes. Finally the section was washed for 2 minutes in buffer, and mounted in buffer, the coverslip edges being sealed with nail varnish. The sections were examined on a Zeiss fluorescence microscope under incident untraviolet illumination and phase contrast.

C. RESULTS

1. Adults.

(a) Anatomy (Fig. 6). The pronephros of adult Coregonus lavaretus is externally indistinguishable from the mesonephros. It comprises the anterior one-tenth of the length of the kidney, and lies immediately posterior to the heart, and dorsal to the swimbladder, gonads, and gut (Plate 4 a-c).

(b) Venous drainage (Fig. 6). The left posterior cardinal vein drains by a number of channels in the mesonephros into the right posterior cardinal vein (Plate 4d), so that as it enters the pronephros, the right vein is much larger than the left. In the pronephros, too, venous drainage is largely towards the right, so that the left lobe contains numerous small veins, rather than one large one (Plate 7). The right duct of Cuvier is much larger than the left, and blood samples were therefore taken from it (p. 9).

(c) Arterial supply (Fig. 6). The dorsal aorta runs along the midline, immediately ventral to the vertebral column and dorsal to the pronephros. A major branch, the coeliaco-mesenteric artery, passes ventrally through the pronephros but gives no branches into it. Immediately after emerging from the pronephros, the coeliaco-mesenteric artery divides into several branches, to the gut and associated organs. The arterial supply to the pronephros is from a number of small arteries around the periphery of the tissue, but whose origin has not been investigated.

(d) Innervation. A large ganglionated sympathetic nerve enters the pronephros on either side, along with the coeliaco-mesenteric artery. These nerves ramify in a complex manner in the pronephric tissue (Fig. 6), their terminal branches being closely associated with the veins (Plate 7). The whole system is much more developed on the right side than on the left.

FIG. 6. Legend.

Serial horizontal sections at approximately 0.5 mm intervals through the pronephros of Coregonus lavaretus, drawn in isometric projection. Anterior to right, dorsal surface uppermost.

The right posterior cardinal vein passes through the pronephros to the right duct of cuvier. The left posterior cardinal vein is a very much smaller vessel, and the left duct of cuvier is correspondingly small. Most of the blood from the left side of the pronephros drains into the right posterior cardinal vein.

Adrenocortical tissue is associated with the posterior cardinal veins and their tributaries on both sides of the pronephros, though the greatest concentration is on the right side.

Chromaffin tissue occurs in isolated clumps on the right posterior cardinal vein (levels C to G), but a small amount is also present on the left side (level G).

The coeliaco-mesenteric artery enters the pronephros dorsally, and passes through it without branching, separated from the haemopoietic tissue by a circum-arterial zone of fat and connective tissue.

Two ganglionated nerves enter the pronephros dorsally, close to the coeliaco-mesenteric artery. The left nerve divides into 3 trunks in the haemopoietic tissue: The right nerve divides into 5; 3 trunks in the circum-arterial zone, and 2 in the haemopoietic tissue (level A).

Left nerve. Of the 3 trunks of the left nerve, the posterior trunk branches towards the right posterior cardinal vein in the region of maximum chromaffin concentration (level C). The median branch fuses with the main trunk of the right nerve in the circum-arterial zone (levels C, D). The anterior trunk continues almost to the ventral surface of the pronephros, where it ramifies into the only concentration of chromaffin tissue in the left side of the pronephros (level G).

Right nerve. The 3 trunks in the circum-arterial zone (level A) fuse to a single large trunk (level B). This single trunk divides into 2 (level C). The larger trunk gives a branch to the right posterior cardinal vein, incorporating the posterior of the 2 trunks of the right nerve in the haemopoietic tissue, in the region of maximum chromaffin concentration (levels C, D). The 2 trunks in the circum-arterial zone continue ventrally, incorporating the median trunk of the left nerve (level D) as described above. A further branch arises (levels E, F) incorporating both the trunks in the haemopoietic tissue, and ramifying in the anterior region of the right posterior cardinal vein where there is a smaller concentration of chromaffin tissue. The two trunks in the circum-arterial zone continue through the pronephros into the coelom (level H), but their courses beyond this point have not been studied.

(only the major branches of the nerves are shown in Fig. 6; minor ramifications can be seen in sections close to the veins and the chromaffin tissue).

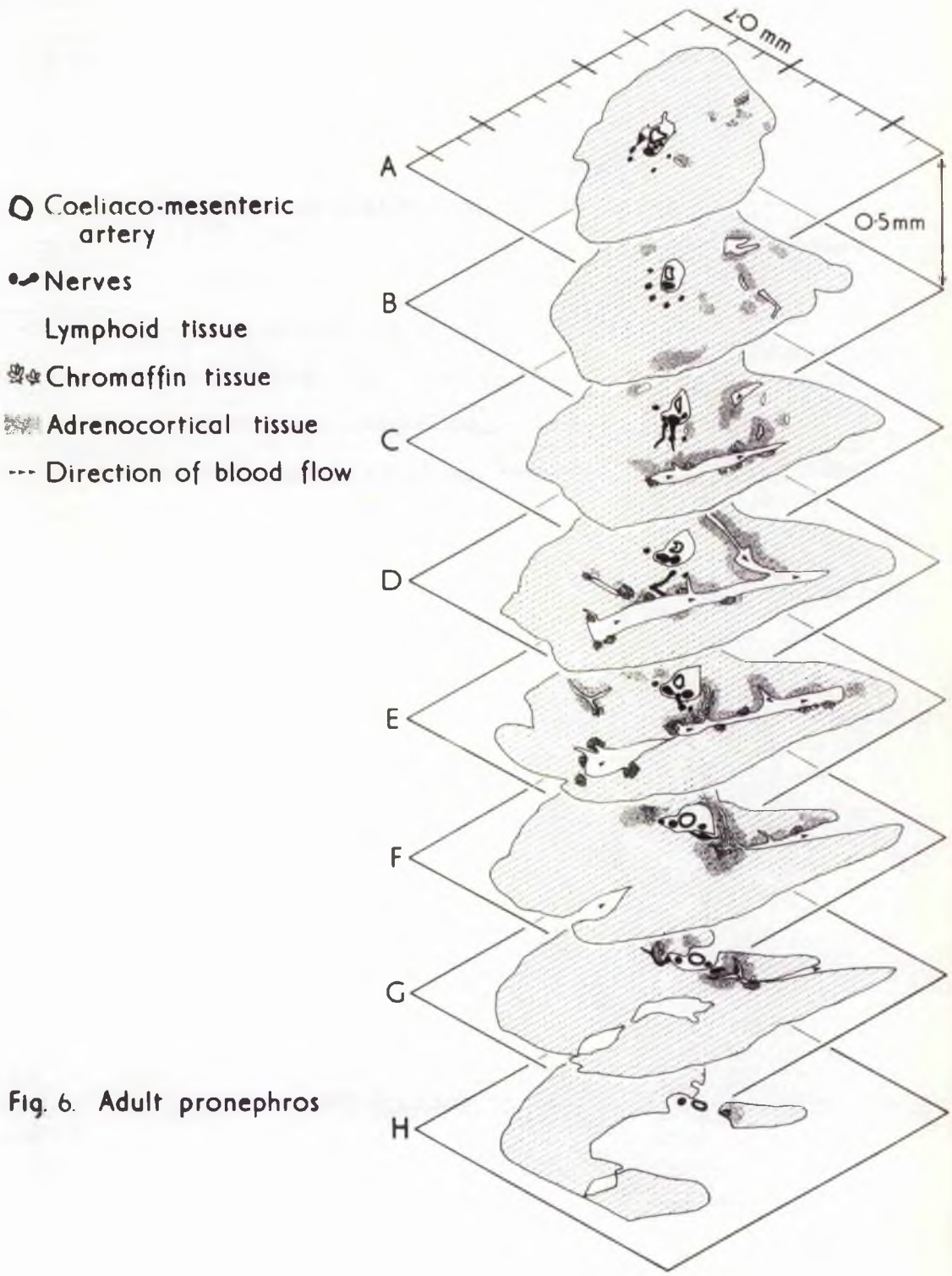


Fig. 6. Adult pronephros

(e) Adrenocortical tissue. The adrenocortical cells occur round the right posterior cardinal vein and its main tributaries and, though less abundantly, round the veins of the left side of the pronephros. Round small venules, the tissue may be only one layer thick, but round larger veins several layers exist (Plate 5a). These were the only cells to give a purple reaction with the 3-β-ol dehydrogenase technique (Plate 6 a-d). Immunostaining with antisera to aldosterone and deoxycorticosterone was ineffective. Antiserum to cortisol gave inconsistent results; on one occasion fluorescence was observed round large veins, where phase contrast revealed adrenocortical cells.

(f) Chromaffin tissue. Staining with Masson's trichrome, especially after fixation in Orth's fixative (p. 22) revealed 2 secretory cell-types. Amongst the adrenocortical tissue occur nests of palely-staining cells, often elongated and curved in shape (Plate 5b). These are concentrated close to the wall of the main right posterior cardinal vein, with a few groups on main tributary veins. Their location on the veins coincides with the main mass of terminations of the sympathetic nerves. On the left side, into which there is little sympathetic nerve branching, there are correspondingly few chromaffin cells (Plate 7). None of the dichromate-based histochemical techniques stained the chromaffin cells specifically, though Orth's fixation may have enhanced the distinction between chromaffin and adrenocortical cells when stained in Masson's trichrome. The Falck and Owman technique, however, revealed nests of fluorescence round veins, in positions which accord with normal histological observations of chromaffin cells (Plate 8 a-b). Unfortunately, the fluorescence was somewhat diffuse, probably because the tissue was at room temperature during freeze-drying, so that it was not possible to match the areas of fluorescence specifically with presumptive chromaffin cells.

2. Powan Fry.

The newly-hatched larva of Coregonus lavaretus has a functional pronephros. There is no mesonephros, though the posterior cardinal veins are conspicuous. Mesonephric tissue appears in the 2-month old fish. Adrenocortical cells appear in the pronephros at 3 months and are abundant by 6 months (Plate 9d). By 9 months the adult pattern of venous, arterial, and nerve supply is established (Fig. 7, Plate 9 a-d). Chromaffin cells, however, are not distinguishable.

3. Other salmonids.

Comparative histological studies were made of pronephroi of Salmo salar (Atlantic salmon - anadromous), Salmo trutta trutta (sea-trout - anadromous), Salmo trutta fario (brown trout - freshwater), Salmo gairdnerii (rainbow trout - freshwater), Salvelinus willughbii (char - freshwater), and Osmerus eperlangus (smelt - marine). Pronephroi from at least 2 specimens of each species were routinely fixed, embedded and stained (pp. 10, 19).

The basic anatomy of the pronephroi of all species except the smelt was as in Coregonus. The adrenocortical tissue is concentrated in the antero-lateral region, around the posterior cardinal veins and their tributaries. In the trout and salmon the cells are in irregular clumps (Plate 10 a, b, d), but in the char they are in regular collars 1 - 3 layers thick, around the veins (Plate 10c). The latter arrangement has been described in the immature salmon (Robertson and Wexler, 1959).

The pronephros of the smelt differed from the other species studied. There are two separate lobes of pronephric tissue connected by a single transverse strand of lymphoid tissue (Plate 10e). The adrenocortical cells surround the main posterior cardinal vein, but not its tributaries, in a

FIG. 7. Legend

Serial sagittal sections (slightly oblique to the dorso-ventral plane) at approximately 175 µm intervals through 9-month-old Coregonus lavaretus, drawn in isometric projection. Anterior to right. Level A is to the right of the animal's mid-line, Level I is to the left of the mid-line.

A. Sections of right anterior cardinal vein and right duct of cuvier.

B. Right anterior and posterior cardinal veins join right duct of cuvier.

C. Pronephric tissue round right posterior cardinal vein; sinus venosus and atrium.

D, E. Pronephros extends posteriorly; atrium; ventricle; bulbus anteriosus and ventral aorta.

F, G, H. Posterior cardinal vein; coeliaco-mesenteric artery passes ventrally through pronephric tissue.

I. epatic vein enters sinus venosus.

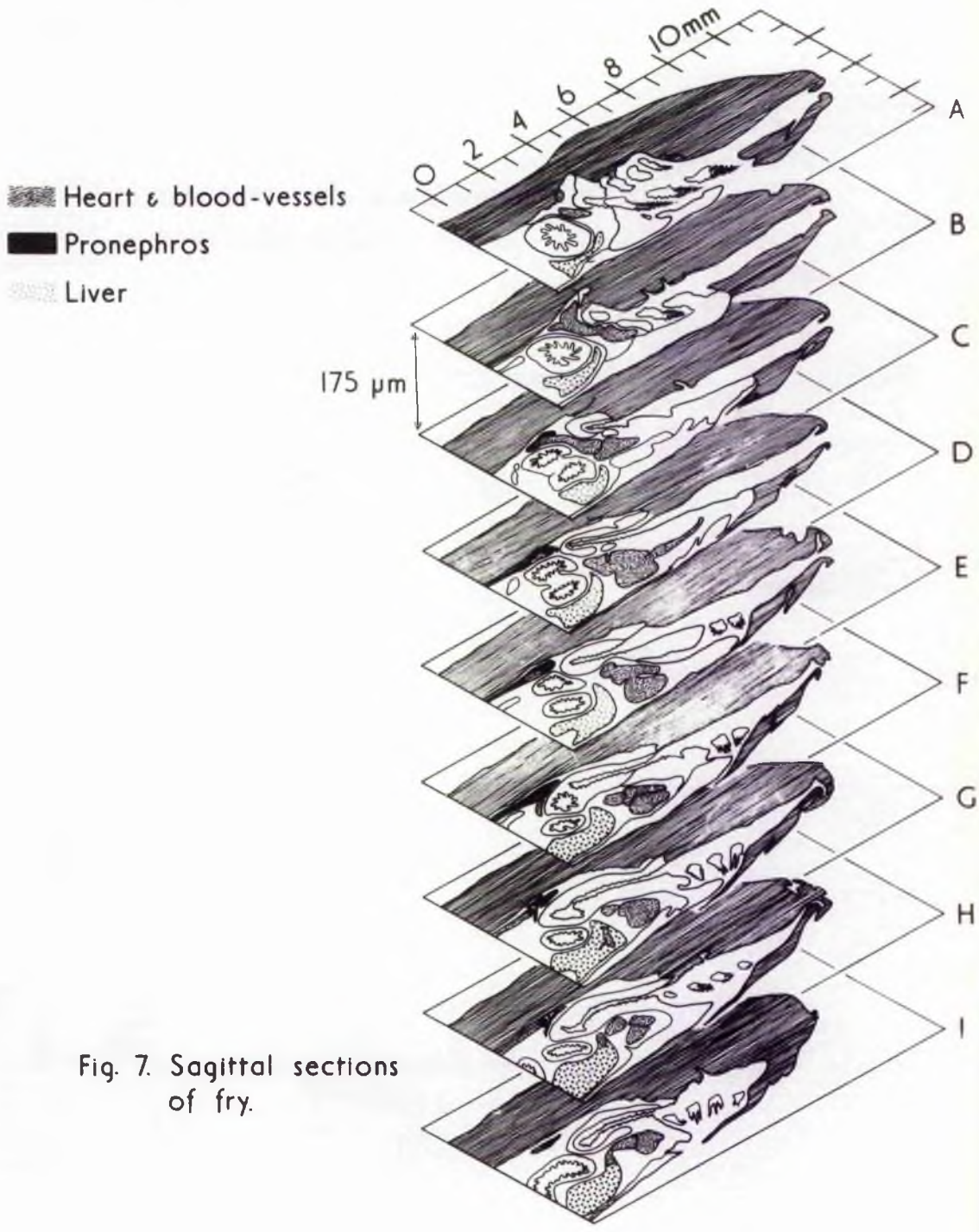


Fig. 7. Sagittal sections of fry.

band 1 - 3 cells wide, and are sparser than in the other salmonids (Plate 10f). A similar arrangement has been described in Phoxinus phoxinus, the european minnow (Scott, 1963).

Chromaffin cells could be found only in the salmon (Pl. 10h), brown trout (Plate 10g), and sea-trout. As in the powan, they are fewer in number than the adrenocortical cells, and lie close to the walls of the posterior cardinal veins. They tend to be located more posteriorly in the head-kidney than the adrenocortical cells. The Falck and Owman technique was applied to the rainbow trout, with the same result as in the powan. Nests of cells fluoresced in positions appropriate to chromaffin cells, but the diffuseness of the fluorescence made it impossible to identify individual cells (Plate 8d, e). The nerve ganglion also fluoresced.

C. DISCUSSION

The pronephros of Coregonus lavaretus contains both adrenocortical and chromaffin tissues. The former is the more abundant, and its identification is established on the basis of the presence of 3-β-ol dehydrogenase. Moreover, the adrenocortical tissue reacts histologically (by change in nuclear diameter) to stress at the same time as histological stress reactions appear in the adrenocorticotropic cells of the pituitary (pp. 109-10). Immunostaining provides no additional evidence of the identity of the adrenocortical cells. The single occasion on which cortisol antiserum gave fluorescence might be explained on the basis of a pronephros having a higher than usual concentration of hormones. Generally, however, the adrenocortical tissue does not accumulate steroids, so that the levels at any given time are low, and presumably insufficient to give a recognisable response.

The identification of the chromaffin cells is less well established. The techniques based onthe classical chromaffin reaction were unrevealing, in some cases because no differential staining occurred, in others because the histological quality of the preparations was so poor that it was impossible to distinguish the presumed chromaffin cells from the rest of the tissue. The latter is a much more serious problem in teleost than in mammalian studies, as a reaction in the mass of the mammalian "adrenal medulla" is very obvious, and the location of the reacting cells is not in doubt. In teleosts, however, reactions may be visible but unless the histological standard is good it is impossible to decide whether it is in fact occurring in the presumed chromaffin cells, or in other cells also scattered about the pronephros. The fluorescence method for biological amines suffered from this defect in that nests of fluorescence were visible in the walls of the posterior cardinal veins, in positions similar to those of the presumed chromaffin cells as seen in Masson-stained preparations, but to equate a specific area of fluorescence with a specific nest of cells was not histologically possible.

The main ramifications of the sympathetic nerves occur in the region of the presumed chromaffin cells, which supports their identification. Under the light microscope it was not possible to determine whether the nerve endings were in fact on the chromaffin cells themselves, or on the associated veins.

The cumulative evidence, however, is that the identification of adrenocortical and chromaffin cells is correct. However, an electron microscope study would clarify the relationship between the sympathetic nerves and the chromaffin cells. Freeze drying at lower temperatures should improve the quality of the technique (p. 26). It is possible that application of the

peroxidase-enzyme antibody technique (Nakane and Pierce, 1967) for locating hormones would prove more sensitive than conventional immuno-staining, and make possible immunological identification of the adrenocortical cells.

CHAPTER 3.

PLASMA CORTICOSTEROIDS IN *Coregonus lavaretus* AND RELATED SALMONIDS

A. INTRODUCTION

1. Review of previous corticosteroid identification in teleosts.

Few corticosteroids have been positively identified in teleosts. Previous findings and techniques employed have been extensively reviewed (Chester Jones *et al.*, 1969; Idler, 1972) and the reliability of the methodology and identifications has been assessed (Idler, 1972). It is possible to distinguish identifications which are positive or which are only tentative or unsatisfactory. The principles of identification of steroids are discussed in detail by Brooks *et al.* (1970). The following is a summary of the corticosteroids so far identified in teleosts.

(a) *In vitro* analyses: Many investigations have involved *in vitro* studies on the adrenocortical tissue to determine steroid production from exogenous precursors and also, in fewer cases, steroids from endogenous precursors in the tissue. Quantitative assessment of the results are difficult, partly due to varying incubation conditions and, also, because of the diffuse nature of teleost adrenocortical tissue, incubates may contain different amounts of extraneous tissue making direct comparison of results impossible.

Several corticosteroids have been identified by employing these *in vitro* techniques. The principal products found were 17-hydroxylated C_{21} steroids. Cortisone and cortisol have been identified from endogenous precursors in several teleosts, usually indicating cortisol as the major product (Sandor *et al.*, 1966, 1967; Nandi and Bern, 1965; Leloup-Hatey, 1964b; Arai, Tajima and Tamaoki, 1969). Most other steroids have been identified following addition of exogenous precursors.

11-deoxycorticosterone (DOC) has not been reported as a product of endogenous precursors other than by Sundararaj and Goswami (1969) working on head-kidney incubates of Heteropneustes fossilis. Using radioactive exogenous precursors such as (^{14}C)acetate, pregnenolone and progesterone, DOC has been identified in Salmo gairdneri, the rainbow trout (Arai, Tajima and Tamaoki, 1969) and in powan (Whitehouse and Vinson, 1973).

Corticosterone has been tentatively identified in several species from endogenous and exogenous precursors (Leloup-Hatey, 1966; Arai, Tajima and Tamaoki, 1969; Idler, 1972).

11-deoxycortisol was isolated by Sandor et al. (1966, 1967) after precursor addition to head-kidney incubates of Anguilla anguilla but their results were not confirmed by further investigation on the same species (Leloup-Hatey, 1966). Powan head-kidney tissue has been shown to be capable of 11-deoxycortisol synthesis from exogenous precursors (Whitehouse and Vinson, 1973).

Aldosterone has not been identified from endogenous precursors in head-kidney incubates. Most studies have also failed to indicate the presence of aldosterone following addition of exogenous precursors. However, the latter methods have permitted identification of this steroid in Clupea harengus (Truscott and Idler, 1968). An earlier study (Phillips and Mulrow, 1959) identified aldosterone in tissues of Fundulus heteroclitus but conclusions from this work were questionable as control tissues yielded higher aldosterone levels than the head-kidney tissues, and methods of steroid identification were inadequate. There are further reports of aldosterone production by head-kidney incubates of Labeo rohita, Cirrhina mrigala and Catla catla (Roy, 1964) but again its identification is only tentative.

Results from *in vitro* studies cannot be used to describe the situation *in vivo*. The use of exogenous precursors for identifying steroid formation in the tissue does not provide proof that these steroids are produced by the tissue *in vivo*, but merely that the tissue possesses the enzyme systems necessary to produce the steroid from a given precursor. In summary, it has been demonstrated that the enzyme systems necessary for C_{17}-hydroxylation, C_{21}-hydroxylation and 11β-hydroxylation (Fig. 2) are present in teleost adrenocortical tissues, though *in vivo* secretion of the corticosteroids formed has not been established by these methods. It is therefore essential to investigate the steroids *in vivo*.

(b) *In vivo* determination of plasma corticosteroids provides a more direct method for identification of corticosteroids in teleosts. Though studies were begun in 1957 (Bondy, Upton and Pickford, 1957; Chester Jones, 1957) confirmation of the identity of few of the steroids has since been confirmed. Techniques available for measuring plasma steroids are discussed in detail later (pp. 42-48) together with advantages and disadvantages of the various methods. The adaptation of these techniques for use on teleost plasma have been reviewed by Idler (1972). Recent methodology has made it necessary to amend early reports on steroid levels and identifications in teleosts. Most work has been carried out on salmonid species (Phillips, Holmes and Bondy, 1959; Hane and Robertson, 1959; Leloup-Hatey, 1964a, b; Hane *et al.*, 1966) and on the remotely related eels, *Anguilla* spp. (Leloup-Hatey, 1964b; Bradshaw and Fontaine-Bertrand, 1968; Butler *et al.*, 1969; Ball et al., 1970).

Cortisol and *cortisone*, as *in vitro* studies suggest, are the major corticosteroids identified in these *in vivo* investigations.

17α-hydroxyprogesterone, 17α, 20β-dihydroxyprogesterone and 20β-dihydrocortisone were found in the plasma of post-spawned Oncorhynchus nerka (Idler, Ronald and Schmidt, 1959; Idler, Fagerlund and Ronald, 1960; Idler, Schmidt and Ronald, 1962).

Corticosterone has tentatively been identified (Phillips, Holmes and Bondy, 1959; Idler, Schmidt and Ronald, 1960; Leloup-Hatey, 1964b). However, chromatographic isopolarity has often been the basis of identifying corticosterone but it is known that corticosterone and 11-deoxycortisol are not readily separated chromatographically. 11-deoxycortisol was found in plasma of Oncorhynchus nerka (Idler, 1960) though identification was only by isopolarity with authentic steroid and by U.V. absorption spectra.

11-deoxycorticosterone (DOC) was isolated and quantified by Chavin and Singley (1972) in one sample of a serum pool from Carassius auratus. Identification was based on chromatographic behaviour of steroids and derivatives compared with authentic compounds. The recorded DOC level was very high compared to levels of DOC in other vertebrates (p. 79).

Aldosterone as a component of normal secretion in teleosts is subject to dispute. Its presence in plasma has been reported only 3 times. The report of Phillips, Holmes and Bondy (1959) is questionable, as the steroid, which was from Oncorhynchus nerka, was identified only by chromatographic mobility. A steroid with isolopolarity to aldosterone was present but it could not chemically be identified as aldosterone (Idler, Ronald and Schmidt, 1959). However, aldosterone has been identified more critically in three out of ten samples from Clupea harengus (Truscott and Idler, 1969), though the same technique did not identify aldosterone in other teleost species including Salmo salar. The only other report of aldosterone is on Carassius auratus, in which Chavin and Singley (1972) estimated 110 ng / 100 ml. serum,

assaying only one sample from a serum pool. As in the case of their results for the DOC level, the level of aldosterone was very high compared to other vertebrate levels (human levels range from 4 - 18 ng/100 ml.).

Thus, it can be concluded that identification of most of the above corticosteroids in teleosts is still not confirmed. In the past, techniques have been unable to detect low levels of circulating steroids. Even with the development of recent, highly sensitive techniques the small volumes of plasma obtainable from most teleosts presents problems. It is also difficult to establish normal levels of circulating steroids in teleosts, where overcoming problems caused by catching and maintenance techniques, in obtaining fish from their natural environment, is difficult and these problems are further discussed in Chapters 1 and 5.

Aim of this section of the project.

In the present study, gas-liquid chromatography (GLC) was employed for three main purposes,

(a) to provide a fast reliable, qualitative and quantitative estimation of plasma corticosteroids in powan before developing saturation techniques for measuring cortisol and DOC in powan plasma (Chapter 4). This was necessary to establish the ratio of both of these steroids to other corticosteroids in the plasma, and to give an indication of the likely range of cortisol and DOC levels. The latter enables optimal standard curves to be produced for the saturation analysis techniques before assaying the powan samples collected.

(b) to confirm the specificity of the saturation analysis techniques. Although GLC requires large volumes of plasma as well as elaborate apparatus,

specificity is easier to establish by a more physicochemical method such as GLC (Fraser et al., 1974), than by saturation analysis methods.

(c) to compare corticosteroid levels in the plasma of the powan with levels in the plasma of other related salmonids. In particular, migrating species such as Salmo salar and Salmo trutta trutta were investigated where mineralocorticoid levels might be expected to differ from those of the freshwater, non-migratory powan.

B. METHOD

1. Plasma samples.

Plasma was collected from gill-netted powan in May. Separate pools of male and female plasma were obtained from groups of 25 fish (as described on p. 9), keeping plasma from different sexes separate, samples were also taken from seine-netted Salmo salar, the salmon, in the freshwater phase before spawning; from seine-netted Salmo trutta trutta, the sea-trout, also in freshwater before spawning, and from gill-netted Salmo trutta fario, the non-migratory freshwater brown trout. These three species were also obtained in late May and plasma samples from 2 individuals were pooled in each case.

2. Gas liquid chromatography (GLC) multiple steroid analysis.

10 ml. plasma samples were halved and extracted with 10 vols. of dichloromethane. A neutral extract was obtained by an alkali-acid-water wash (P. 53). These analyses were carried out in collaboration with Mr. P. Mason (M.R.C. Blood Pressure Research Unit, Western Infirmary, Glasgow) who carried out the GLC measurements using the method of Mason and Fraser (1974). The method is based on derivative formation in conjunction with paper chromatographic purification steps with final injection into a

gas-liquid chromatograph (Pye 104, Pye Unicam) fitted with a ^{63}Ni electron capture detector and a bypass valve (Wilson and Fraser, 1971). The steroids investigated were cortisol, corticosterone, 11-deoxycortisol, DOC, 18-hydroxy DOC, and aldosterone.

C. RESULTS

Cortisol, corticosterone, DOC, 11-deoxycortisol, 18-hydroxy DOC and aldosterone are normal secretion products in Coregonus lavaretus (Table 2), Salmo salar, Salmo trutta trutta and Salmo trutta fario (Table 3). The levels are well within the limits of detection of the GLC method, which are 0.45 pg for cortisol, 2.5 pg for corticosterone, 1.56 pg for DOC, 0.33 pg for 11-deoxycortisol, 1.68 pg for 18-hydroxy DOC and 1.44 pg for aldosterone. The reliability, specificity, accuracy and precision of the GLC method have been critically established (Mason and Fraser, 1974).

TABLE 2. Plasma corticosteroids of Coregonus lavaretus, assayed by gas-liquid chromatography. Each sample consists of a pool from 25 gill-netted fish.

Plasma corticosteroid	February		June	
	♂	♀	♂	♀
Cortisol µg/100 ml	7.8	9.2	6.7	8.0
11-deoxycorticosterone (DOC) ng/100 ml.	6.0	7.0	4.0	4.0
Corticosterone ng/100 ml.	37.0	15.0	87.0	23.0
11-deoxycortisol ng/100 ml.	206.0	258.0	105.0	67.0
18-hydroxydeoxycorticosterone (18-hydroxy DOC) ng/100 ml.	87.8	17.6	14.9	25.2
Aldosterone ng/100 ml.	1.9	8.0	2.0	2.0

TABLE 3. Plasma corticosteroids of Salmonidae, assayed by gas-liquid chromatography. Each sample pooled from two fish.

Plasma corticosteroid	Salmo salar, salmon, ♂, seine-netted	Salmo trutta trutta, seatrout, ♂ seine-netted	Salmo trutta trutta, seatrout ♀ seine-netted	Salmo trutta fario, brown trout, ♀, gill-netted
Cortisol µg/100 ml.	0.5	2.4	1.6	31.4
11-deoxycorticosterone (DOC) ng/100 ml.	5.9	12.2	7.9	21.6
Corticosterone ng/100 ml.	62.0	22.0	49.0	36.0
11-deoxycortisol ng/100 ml.	27.0	70.9	133.3	543.2
18-hydroxydeoxycorticosterone (18-hydroxy DOC) ng/100 ml.	17.2	5.7	20.6	23.7
Aldosterone ng/100 ml.	15.0	1.8	8.0	6.6

D. DISCUSSION

Cortisol as in previous studies of teleost corticosteroids is by far the most abundant steroid. The presence of cortisone has not been investigated in this study, though, on the basis of previous studies, it might be expected to be present in relatively high concentrations.

17-deoxysteroids (corticosterone and DOC) are present in all the species studied. Previously, doubt existed as to whether teleost adrenocortical tissue in fact secretes the 17-deoxysteroids. Although *in vitro* studies have shown that the enzymes required for their synthesis exist, the same enzymes are involved in the synthesis of cortisol; and since the 17-deoxysteroids are present in low concentrations in the plasma they remained undetected by early methods. It was questioned whether 17-deoxysteroids accumulate for secretion at all if a strong 17 α-hydroxylase is present (Fig. 2), (Idler, 1972). The present study establishes the existence of corticosterone and DOC in the powan and other salmonids investigated, and the range of concentration of the two hormones is similar to that in human plasma.

11-deoxycortisol is also present in the powan and *Salmo* species studied. Among the latter, only the brown trout were gill-netted and it is noteworthy that 11-deoxycortisol was high in this species, as were cortisol and DOC. This corresponds with the rise in cortisol and DOC in powan caught by gill-netting and maintained in aquaria (Chapter 5). Corticosterone, 18-hydroxy DOC and aldosterone did not show such comparatively high levels in the brown trout.

Aldosterone is present in the plasma of all the species in the present study. Its existence, and even the existence of 18-hydroxylating enzymes in teleost plasma has been disputed. The pathway of aldosterone synthesis in humans is:

More work is clearly required before any conclusions can be drawn as to the role of aldosterone as a mineralocorticoid in teleosts, but the comparatively high level of aldosterone in the salmon (15 ng/100 ml. - Table 2) is noteworthy. This fish was in the early freshwater stage of its anadromous migration. The non-migratory powan (Table 2) and brown trout (Table 3) had low levels. The phase of migration of the sea-trout was uncertain; aldosterone was low (Table 3). Unfortunately, there is no evidence of the role of aldosterone in teleosts comparable to the sodium regulating systems which exist in mammals, but it would not be unreasonable to postulate a link between teleost aldosterone and osmotic stress during migration.

It is clear that more detailed information is essential before the role of each of the adrenocorticosteroids can be understood, and each teleost species must be considered in its own right. *In vivo* studies are clearly more direct than *in vitro* studies, and are to be preferred for this reason. In the present study, two corticosteroids have been selected for detailed study in a single species, *Coregonus lavaretus*. The steroids selected are (a) cortisol, because it is the most abundant, and (b) DOC, because, although it is a mineralocorticoid in mammals, it has been suggested that it may be involved in a critical reproductive phase, ovulation, in teleosts (Goswami and Sundararaj, 1971a, b). The techniques employed in measuring these two hormones are detailed in chapter 4 and the results in chapter 5.

CHAPTER 4.

SATURATION ANALYSIS TECHNIQUES FOR ASSAYING PLASMA CORTISOL AND 11-DEOXYCORTICOSTERONE (DOC) IN THE POWAN

A. GENERAL INTRODUCTION

Preliminary analyses by gas-liquid chromatography (Chapter 3) indicated that powan cortisol and DOC occur in similar levels in the plasma to those found in normal human peripheral plasma. However, unlike humans, availability of plasma from individual powan is limited (p. 9), necessitating the use of small volumes for analysis on individual plasma samples. Hence, sensitivity of detection represents the main limiting factor in choosing a suitable technique for analysis. Also, it must be borne in mind that fish plasma will contain many compounds of triterpenoid type or origin (of similar structure to corticosteroids) secreted, for example, by the adrenocortical tissue, gonads and liver, several of which will be present in higher concentrations than those of cortisol and DOC. Thus, methods must be capable of distinguishing cortisol and DOC from these closely related substances, that is, they must be specific. Finally, for the studies proposed (Chapter 5), where random samples of a relatively large fish population have been taken, large numbers of samples must be analysed in order that the statistical significance of the results may be adequate. Thus, to keep the project within manageable proportions, the methods chosen for plasma analyses should be as simple and practicable as possible without jeopardising sensitivity or specificity.

1. Techniques available for measuring steroids.

A number of features of the corticosteroid molecule and its chemical and biochemical behaviour have been used as bases for methods of quantitation. Techniques which have been applied include:

(a) U.V. absorptiometry: The 3-en-4-one grouping in the A ring of corticosteroids, some androgens and progesterone, absorbs U.V. light at 241 nm. However, detection is limited to quantities of 50 ng or above, even when pure standards are used (Short and Levett, 1962). Specificity is poor and interference from reagents may also be a problem. Nevertheless, this corticosteroid property has been employed qualitatively to demonstrate specificity together with other more specific tests (p. 60).

(b) Colorimetry: The α-ketol side chain of corticosteroids has reducing properties which have been employed in quantitative chromogenic reactions. Based on these reactions are assay methods depending on the production of molybdenum blue (Heard and Sobell, 1946) or the reduction of tetrazolium salts (Mader and Buck, 1951) to form coloured formazans. Neither of these approaches is sensitive enough for the purpose of this study.

The presence of the 17α, 21 dihydroxy-20-one configuration in the cortisol molecule allows the formation of a yellow phenylhydrazone by reacting with phenylhydrazine in concentrated sulphuric acid (Porter and Silber, 1950; Silber and Porter, 1954). Again, even under ideal conditions, 5 - 10 ml. of human plasma are required for each estimation, making it of little use for a study on pewan plasma. However, it was found of qualitative value in estimation of specificity (p. 60).

(c) Fluorimetry: Solutions of some corticosteroids, in ethanolic sulphuric acid fluoresce when excited with light at 470 nm, emitting light at 530 nm (Sweat, 1955). Very small quantities of pure steroid can be detected in this way and methods requiring only 50 µl of human plasma have been based on this technique. Simple fluorimetric methods have found widespread clinical use (Mattingly, 1962). Unfortunately, the technique is prone to non-specific interference from contaminants in biological fluids and

even from inorganic and organic reagents. As much as 30% of the acid-induced fluorescence may be non-specific (James, Townsend and Fraser, 1967).

(d) Isotope derivative methods.

A number of substituents may be attached to the steroid molecule, the simplest of which is the acetate group, to form the steroid acetate (e.g. cortisol-21-acetate). If acetic anhydride, radioactively labelled at known specific activity, is used, then the mass of the derivative can be calculated from the content of radioactive isotope. Sensitivity depends solely on the specific activity of the reagent. (^{3}H) Acetic anhydride is available at a Ci/mM. Several methods have been based on this technique (Coghlan and Scoggins, 1967; Fraser and James, 1968; Odie, Coghlan and Scoggins, 1972) incorporating a second isotope in the steroid internal standard (i.e. double isotope derivative technique) which monitors recovery. Unfortunately, although adequate sensitivity might have been obtained in this way, because acetylation is a relatively non-specific manoeuvre, the acetate must be laboriously purified by multiple chromatography stages before assay is possible. This results in a practical capacity too small to cope with the proposed study on powan samples (Chapter 5).

(e) Gas-liquid chromatography (GLC) with electron capture detection: Employing the GLC method (Mason and Fraser, 1974), cortisol and DOC can be detected in sub-picogram quantities by the electron detector (after formation of the 3 enyl-bis-heptafluorobutyrate of DOC and after formation of androstenetrione heptafluorobutyrate from oxidation of cortisol). In addition to this extreme sensitivity, gas-liquid chromatography prior to quantitative detection is a powerful means of purification, giving the technique considerable inbuilt specificity. However, although this method was used for calibration purposes (p. 35) large volumes of plasma are required (5 - 10 ml.) and equipment is expensive and technically complex.

Although unsuitable for use in this project GLC may prove advantageous for use on teleost plasma where larger volumes of plasma are available.

(f) Saturation analysis: Steroids tend to bind to protein surfaces. If a given quantity of protein in solution is incubated with radioactively labelled steroid, an equilibrium is established between the protein-steroid complex (i.e. bound steroid) and steroid free in solution. If unlabelled steroid is now added to this equilibrium, it will compete with the labelled steroid for binding sites on the protein and radioactivity will be displaced in proportion to the mass of unlabelled steroid added. This is the basis of saturation analysis techniques. Requirements are:-

a radioactively labelled tracer of high specific activity,

a specific binding protein,

and a method of separating bound and free steroid components.

Radioimmunoassay is a saturation analysis using an antibody as the binding protein.

Competitive protein binding is a saturation analysis in which specificity is essentially dependent upon the specialised binding properties of a protein.

These principles were used as a basis to establish saturation analysis techniques for assaying plasma cortisol and plasma DOC in the powan.

Separation of bound from free steroid. When equilibrium has been reached following incubation of binding protein, steroid and tracer, to assess the ratio of bound to free steroid, either the bound or the free steroid must be removed. This may be accomplished either by precipitating the protein-steroid (bound) complex, leaving free steroid in the supernatant or by

absorbing or adsorbing the free steroid component, leaving the bound fraction for assessment of radioactive content.

Precipitation of bound steroid. Ammonium sulphate at 50% saturation causes the precipitation of Y-globulins, the fraction containing most antibodies. These insoluble proteins can be removed by centrifugation and the supernatant containing free steroid can be analysed for radioactivity. The technique has been applied to radioimmunoassay (Mayes et al., 1970) but not to competitive protein binding. Another approach has been to produce a second antibody to the Y-globulin fraction to co-precipitate with the bound steroid. The problem of separation can also be averted by converting the antibody to the solid phase (Mikhail, Ferrin and Vande Wiele, 1971; Midgeley, Rebar and Niswender, 1969), separation is merely by decanting. This method has not been applied to competitive protein binding.

Removal of the steroid. Many porous substances act as molecular sieves which allow the entry of small molecules such as steroids while excluding protein molecules which are larger. Charcoal is a good example. Following equilibration of a saturation analysis assay, a small amount of charcoal powder may be added which rapidly absorbs free steroid which can then readily be removed by centrifugation (Herbert et al., 1965). There is some evidence that the activated surface of charcoal particles may cause disruption of a proportion of the steroid protein complex, and may also adsorb some of it which will then precipitate,. Herbert et al. (1965) have recommended coating of the charcoal particles with a dextran lattice and also carrying out the assay at low temperatures (4°C).

The problem of steroid adsorption. It is relevant to consider the effect of the general tendency of polar steroids such as cortisol to adsorb to glass surfaces. This may be particularly serious in relatively pure, dilute solutions such as occur during saturation analysis incubations. Considerable distortion of standard curves may occur (Fraser et al., 1974) if steps are not taken to minimise the effect, distortion which may lead to underestimation of steroid concentrations. The problem is most serious where steroid solutions are evaporated to dryness in the reaction vessel but is relatively easily overcome by siliconising glass surfaces, adding small quantities of non-volatile solvents such as ethylene glycol to prevent complete evaporation and by adding carrier proteins such as bovine or human γ globulin to incubating solutions.

The dextran-coated charcoal technique of separating bound from free steroid fractions has been widely applied to competitive protein binding and radioimmunoassay methods and was chosen for use in this study.

2. Methods of steroid extraction and purification.

The corticosteroid must first be extracted from the plasma and then isolated from those compounds which are likely to interfere with its assay. This is of particular necessity for the competitive protein binding assay which is not sufficiently specific (p. 50) to allow direct estimation in plasma or even in crude lipid extracts of plasma.

(a) Extraction. No comparison of different techniques of extraction was made. In general, a crude lipid extract is made by partition with an organic solvent such as dichloromethane, chloroform or ethyl acetate. In addition to its ideal partition properties when mixed with plasma, dichloromethane has a relatively low boiling point, allowing easy evaporation

B. COMPETITIVE PROTEIN BINDING TECHNIQUE FOR MEASURING ROMAN PLASMA CORTISOL

INTRODUCTION

Fortunately, (^{3}H)-cortisol is available commercially at high specific activity suitable for use as radioactive tracer. As a binding surface, two alternatives exist for cortisol: the natural, circulating corticosteroid binding globulin (CBG), transcortin, which is synthesised in the liver and is present in mammalian plasma or cortisol antibodies.

Cortisol antibodies. Molecules such as cortisol which have a molecular weight of less than 1000 are not antigenic, but if they are covalently linked as haptens to proteins such as bovine serum albumen, antibodies can be induced which bind the hapten with variable but generally high specificity (Erlanger et al., 1957). In recent years, antibodies have been raised to many steroids, including corticosteroids, and these have been used as the specific binding proteins for sensitive and convenient methods of saturation analysis, the radioimmunoassay methods. Strangely enough, fewer attempts seem to have been made to raise antibodies to cortisol than to other corticosteroids such as DOC, aldosterone and corticosterone. Several reasons may account for this. Cortisol is the most concentrated of corticosteroids in man, to whom the majority of studies apply and there is no lack of plasma availability in this species unlike teleosts. The extreme potential sensitivity of radioimmunoassay is not necessary for studies on man. Moreover, cortisol is immuno-suppressive and may generate antibodies less readily than other steroids. Nevertheless, antisera have been raised in sheep and rabbits (Wynne-Roberts, 1974) and used successfully in radioimmunoassay procedures (Ruder, Guy and Lipsett, 1972).

The criteria for the successful production of useful steroid antisera are as yet only vague and anecdotal but it is clear that immunisation frequently requires a lengthy period of several months and success cannot

confidently be predicted. Since cortisol antisera are not commercially available but the alternative binding protein, transcortin (CBG) is readily and freely obtainable (see below) it was decided to investigate the use of this latter protein.

Corticosteroid-binding globulin (CBG). In common with other steroids, a large proportion of the cortisol circulating in blood is bound to a globular protein. In mammals, this protein is 'transcortin' or CBG. Like many other plasma proteins CBG is synthesised in the liver and its production rate is increased by oestrogen administration (Murray, 1967). Therefore, in late human pregnancy, where oestrogen production is high, plasma CBG levels are markedly raised. Human late pregnancy plasma is an abundant and convenient source of binding protein.

The principle of saturation analysis using CBG is usually known as competitive protein binding assay and was introduced by Murphy (Murphy and Pattee, 1964; Murphy, 1969) for the estimation of 11-hydroxycorticosteroid concentrations in human plasma. Such assays are relatively sensitive, requiring only 1 ml. of human plasma. Modification of the conditions of assay were required in order to obtain sufficient sensitivity for analysis of powan plasma. CBG is a less specific binding protein than are cortisol antibodies, binding other corticosteroids such as corticosterone equally well. Thus, preliminary isolation and partial purification of cortisol is necessary.

2. Materials.

Solvents. Dichloromethane (M. and B. Lab. Chemicals) was redistilled in a fractionating column before use. All other solvents were of 'Nanograde' quantity (Mallinckrodt Chemical Works).

Water was twice distilled from all-glass apparatus.

The B_5 chromatography system (Bush, 1961 modified by Mayes et al., 1970) contained benzene:hexane:methanol:water in proportions 9:1:5:2.5.

Scintillator solution (PPO) contained 0.5% 2,5-diphenyloxazole in toluene containing 2% methanol. Toluene (sulphur free, B.D.H. Lab. reagent) was used as supplied.

Tris buffer contained 24.2 g. Tris (Analar, B.D.H. Chemicals, Ltd.), 1 g. EDTA (M. and B. Lab. Chemicals) and 15.5 ml. conc. hydrochloric acid in 1 l. water.

Chromatography paper. (Whatman 2, W. and R. Balston, Ltd.) was washed in a Soxhlet apparatus for at least 24 hrs. and was stored in dust-free conditions.

Steroids. Unlabelled cortisol (Koch Light and Co. Ltd., Colnbrook) was dissolved in methanol and stored at 4°C. (^{3}H)-cortisol (Radiochemical Centre, Amersham) of specific activity 40 - 60 Ci/mM was purified by paper chromatography (B_5 system) before use and stored at 4°C in methanol; for recovery purposes 25,000 cpm (37,500 dpm), equivalent to 120 pg, was added to each plasma sample. A standard solution of (^{3}H)-cortisol containing 5000 cpm/0.1 ml. methanol was also stored at 4°C.

Materials for assay procedure. CBG was obtained from women in the third trimester of pregnancy and stored in aliquots (-20°C) until required. Human γ-globulin (Protein Fractionation Centre, Royal Infirmary, Edinburgh) was also stored at 4°C in Tris buffer (0.5 M, pH 7.0) at a concentration of 2.5 mg./ml.

Ethylene glycol (B.D.H. Ltd., 3% v/v in 0.9% saline w/v) was also stored at 4°C.

Charcoal (Norit-A, Hopkins and Williams, Ltd.) was washed in a Soxhlet apparatus and dried thoroughly to remove traces of methanol. For separating the bound and free steroid components in the saturation analysis (see below) a solution containing 2.5 mg./ml. of charcoal and 1.25 mg./ml. of Dextran T40 (Pharmacia, Uppsala), in Tris buffer, was prepared and stored at 4°C.

Glassware - was cleaned ultrasonically in "Decon" detergent, rinsed several times in tap water, soaked in "Siliclad" solution for 15 mins. (to reduce adsorption of steroid to glass surfaces), rinsed in tap water again several times, rinsed once in distilled water, and oven dried at 40°C overnight.

Assessment of radioactive content was carried out on a Nuclear Chicago Mk I liquid scintillation spectrometer, counting each sample for 10 minutes.

3. Method.

(a) Extraction of steroid from plasma. Pooled plasma (0.1 ml.) diluted with water (0.3 ml.) and containing (^{3}H)-cortisol (25,000 cpm in 0.1 ml. methanol) was extracted with 10 ml. of dichloromethane. After centrifugation at 2,000 rpm for 3 mins. the plasma layer was discarded.

(2 samples of the 25,000 cpm standard solution of (^{3}H)-cortisol were checked for tritium content).

A neutral extract was obtained by washing the residue with 1 ml. N/10 NaOH, followed by 1 ml. N/10 acetic acid and finally with 1 ml. water - centrifuging for 3 mins. 2000 rpm before removing the water layer.

The extract was evaporated to dryness under a stream of nitrogen gas, at less than 35°C(avoiding destruction of steroid). All subsequent evaporations were carried out under these conditions.

(b) Purification of extract. Further purification was achieved by chromatography of the residue on paper. The dry residue was transferred to the origin on the chromatogram in small volumes of dichloromethane which was evaporated under a slow jet of nitrogen. The chromatogram was developed in the modified B_5 system for 16 hrs. (following equilibration in the stationary phase for 1½ hrs.) at 22°C. The paper was dried and the cortisol region was located by means of radioscanning equipment (Panax Ltd.). This region was eluted chromatographically with 5 ml. methanol into a graduated glass tube (fig. 8). The volume of the eluate was reduced to 2.5 ml., the tube was stoppered and left to reach room temperature.

(c) Competitive protein binding assay. 2 aliquots of the 5000 cpm (^{3}H)-cortisol solution were checked for tritium content.

(^{3}H)-recovery of the sample eluate was assessed by counting 0.1 ml. From this information duplicate aliquots of the sample (containing 5000 cpm) were taken for saturation analysis. 0.1 ml. ethylene glycol was added to each sample to prevent adsorption of steroid to the sides of the tube during evaporation).

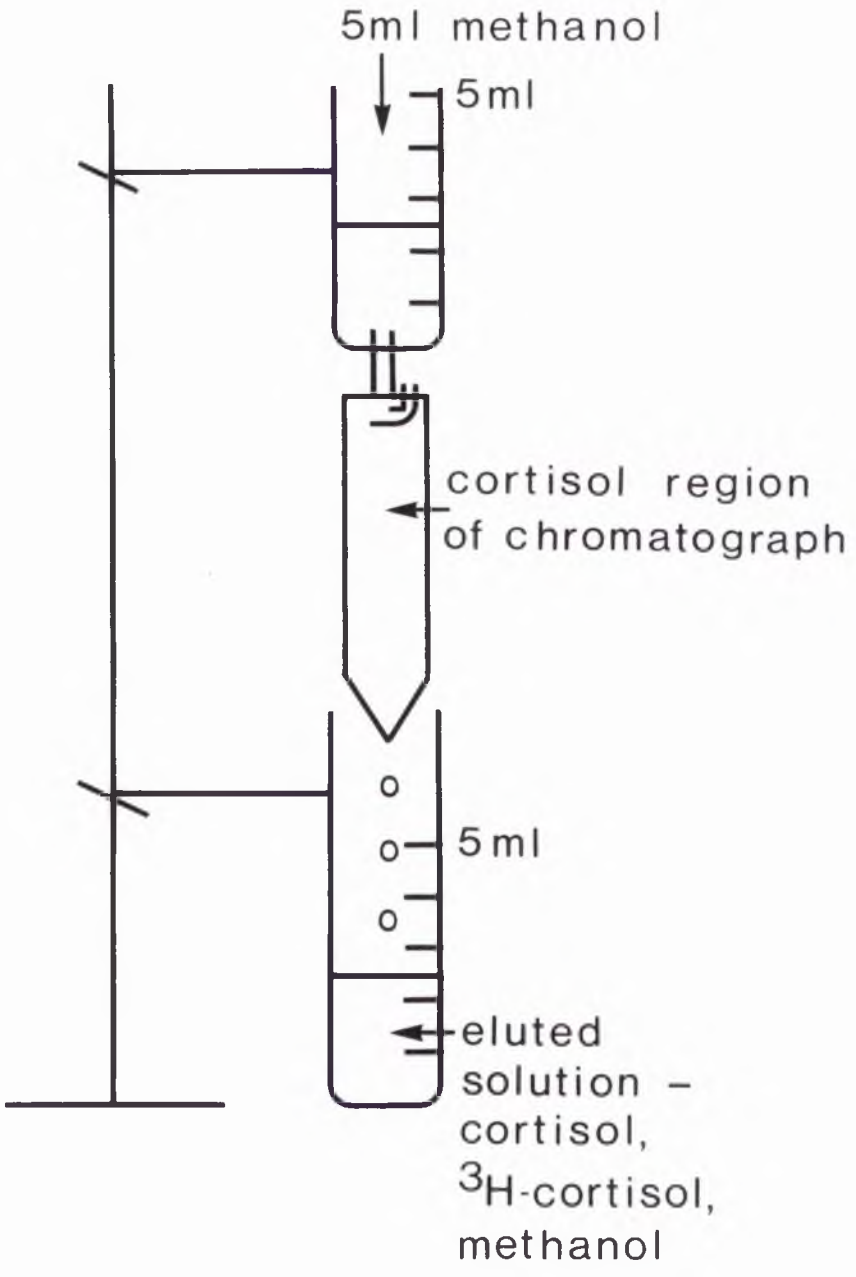


Fig.8. Elution procedure.

Duplicate aliquots of pure unlabelled cortisol, ranging from 0 - 15 ng/0.1 ml. methanol, were treated in the same way. Two extra aliquots of the blank solution (no cortisol) were taken as 'standard blanks'. To each of the standards 0.1 ml. ethylene glycol was added and 5000 cpm (^{3}H)cortisol (in 0.1 ml. methanol).

All samples and standards were evaporated to dryness. Assessment of cortisol concentration was carried out by the competitive protein binding method of Murphy (1967):

Ethanol saline (1 ml.) was added to each tube, mixed and placed in a water bath at 45^{o}C for 5 mins. The tubes were removed from the water bath and after 20 mins. 1 ml. CBG solution was added (0.25% in Tris buffer), mixed and left again at 45^{o}C for 5 min. The tubes were transferred to an ice-bath, after 10 mins. 0.1 ml human Y globulin solution was added. 0.5 ml. dextran coated charcoal solution (keeping the mixture stirring) was added to all tubes except the 2 'standard blanks'. To the latter, 0.5 ml. distilled water was added. All tubes were mixed, centrifuged at 4^{o}C, 10 mins, 2500 rpm. A 0.5 ml. aliquot from each sample was added to 8 ml. scintillator solution and 0.1 ml. methanol (because the standard (^{3}H)-cortisol solutions are in 0.1 ml. methanol). The (^{3}H) content was assessed, this represents the bound fraction of (^{3}H)-cortisol.

TABLE 4: Summary of competitive protein-binding analysis.

Method	Materials	Result
Extraction (of samples)	Methylene chloride + (^{3}H)-cortisol Alkali-acid-water wash	Extracts steroids and separates off plasma contaminants including binding-proteins and phenolic steroids
Purification (of samples)	Modified B_5 paper chromatography system	Separates cortisol from other major plasma steroids
Quantitation (of samples and standard cortisol solutions	1 ml. ethanol-saline, water-bath 45°C, 5 mins. 1 ml. CBG solution, water-bath 45°C, 5 mins. Ice-bath, 10 mins. Ice-bath 0.1 ml. human Y globulin solution 0.5 ml. dextran-coated charcoal Ice-bath further 10 mins.	Dissolves sample for assay (^{3}H)-cortisol and sample compete for binding with CBG. Carrier protein. Also combats loss of steroid due to adsorption Separates bound and unbound steroid by adsorption of unbound fraction

(d) Calculation of cortisol content in plasma sample. The percentage binding of the (^{3}H)-cortisol to the CBG was calculated for each standard as a direct percentage of the cpm of the two standard blanks (to which no charcoal was added, and were taken to represent 100% bound (^{3}H)-cortisol). A standard curve was drawn from the data (fig. 9), plotting percentage binding (^{3}H)-cortisol against ng cortisol/0.1 ml.

In the same way, for the plasma sample, the percentage binding was calculated relative to the two standard blanks. By reference to the standard curve, the values in ng/0.1 ml. for cortisol can be read (x ng). The results were expressed in µg/ml. thus:-

$$\text{ng in 0.1 ml. plasma} = x \cdot \frac{25000}{5000}$$

(as only 5000 cpm of the extract eluate are taken for assay)

$$\therefore \text{µg cortisol/100 ml. plasma} = x \cdot \frac{25000}{5000} \cdot \frac{1000}{1000} = 5x$$

The validity of the method was assessed for specificity, sensitivity, accuracy, precision and reproducibility.

4. Results of assessing validity of competitive protein binding method

(a) Standard curve.

Reproducibility, sensitivity, range. Reproducibility was assessed by analysing ten consecutive standard curves (fig. 9). The same standard cortisol solutions were used throughout. The smallest quantity of steroid which could be distinguished from zero i.e. sensitivity of detection, was taken as that represented by a percent binding at 1.96% standard deviations from the mean of the percent binding at 0 ng on the standard curve. This was calculated to be 0.02 ng. The range of the curve was 0.1 - 50 ng/0.1 ml. plasma (range multiplied by 5 as $^1/_5$ of the extract is taken for saturation analysis).

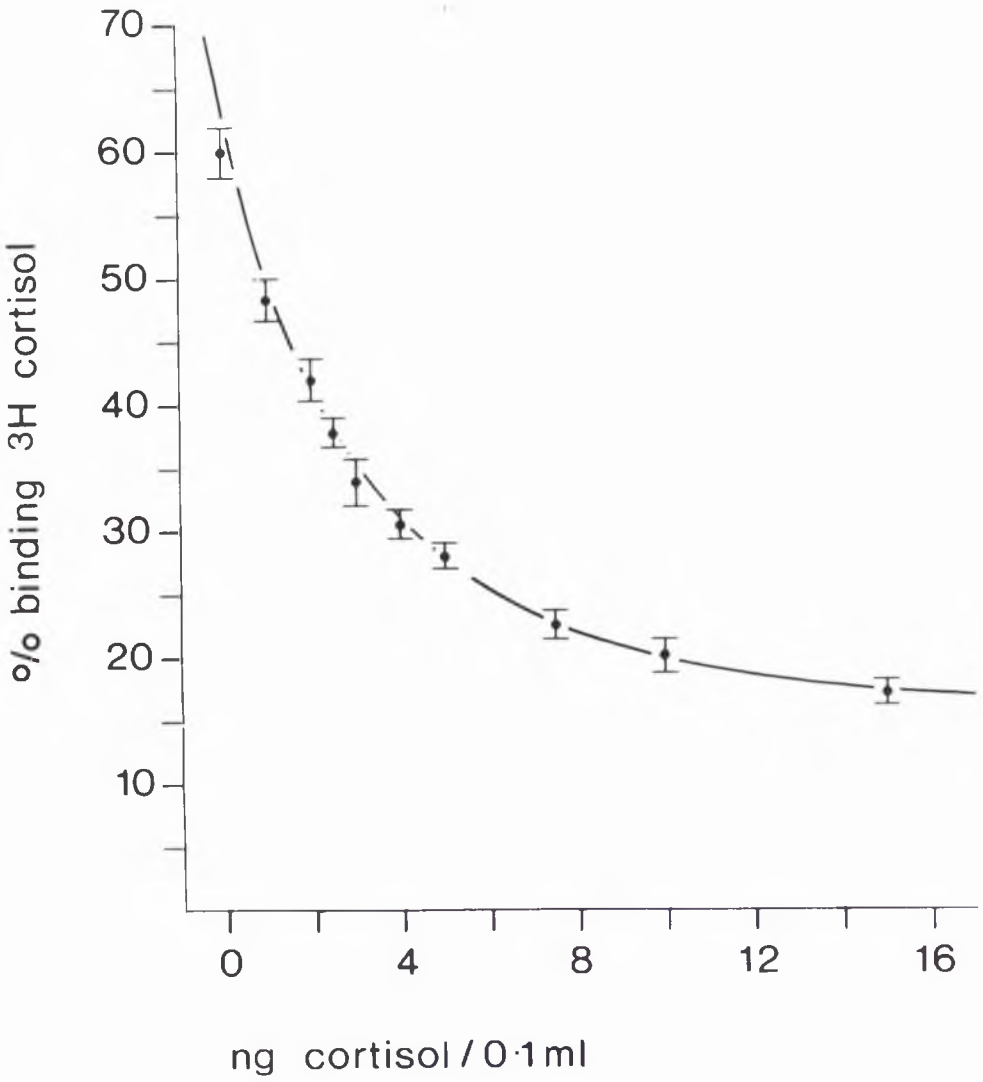


Fig. 9. Reproducibility of standard curve (means ±1 S.D. for 10 consecutive curves).

Effects of volume of incubation. To determine whether solubility or adsorption factors were affecting the standard curve, the volume of CBG solution incubated during the assay was varied and the resulting standard curves were compared. No such effects were observed, and for convenience 1 ml. of CBG solution was used throughout (Fig. 10).

Effects of relative amounts of (^{3}H)-cortisol:CBG concentration. To obtain a standard curve covering the range of cortisol levels to be measured (predicted on the basis of GLC readings and previous investigations on teleost plasma cortisol to be 0 - 50 µg/100 ml. plasma), and with maximum gradient over this range (to increase accuracy), the concentration of CBG solution was varied whilst keeping the amount of (^{3}H)-cortisol constant. Standard curves using 1%, 0.75%, 0.5% and 0.25% CBG concentrations were obtained (fig. 11). The 1% concentration provides a curve suitable for human plasma concentrations, but as higher cortisol levels were likely to be encountered in powan plasma, a steep slope was required over a greater range of the standards. The 0.25% CBG solution produced a suitable curve. Further batches of CBG plasma used in the later part of the study produced similar standard curves at concentrations of 0.3% and 0.25%.

(b) Recovery of (^{3}H)-cortisol and accuracy. High recovery after extraction and chromatography is desirable and recoveries were calculated for 30 samples as 85.8% ± 1.43 (mean ± 1 standard deviation).

Accuracy of the method was determined by adding known quantities of cortisol to a human plasma pool, recoveries ranged from 98 - 110% (Table 5).

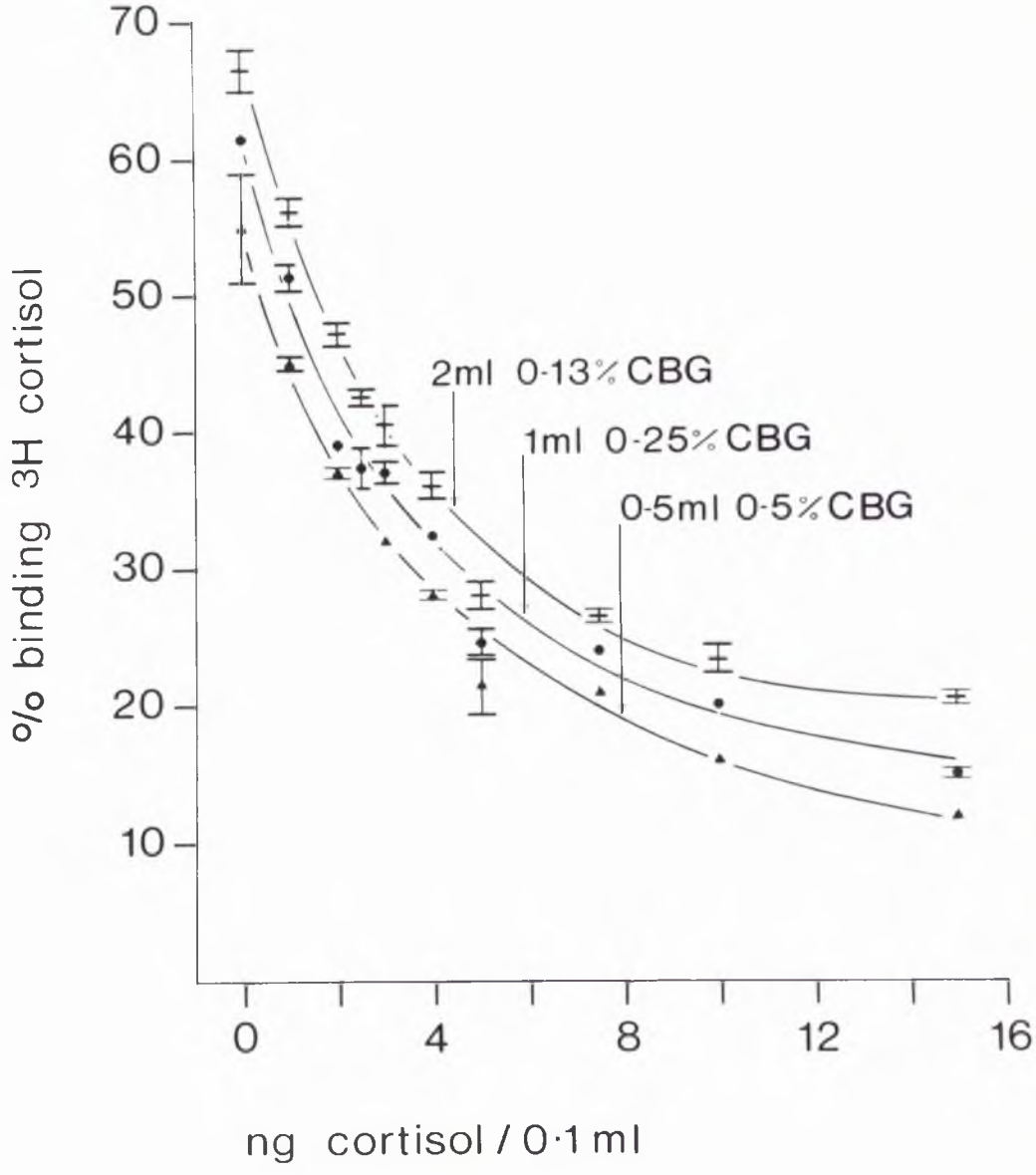


Fig.10. Effects of volume of incubation on standard curve.

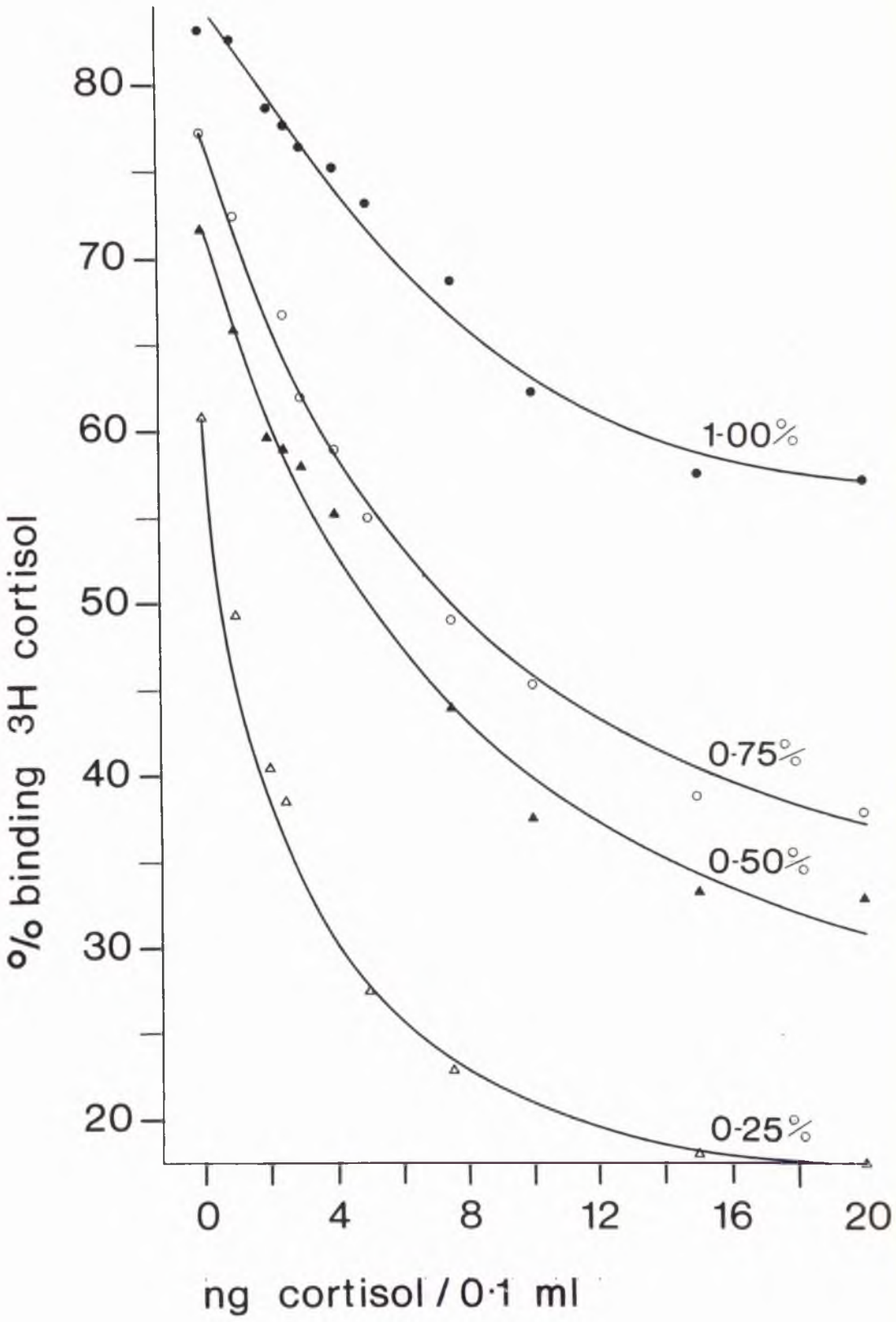


Fig.11. Effect of CBG concentration on standard curve.

TABLE 5. Cortisol level in pooled human plasma with known additions of cortisol.

Sample	Total cortisol	added cortisol recovered	% recovery
blank (water)	0.00		
human plasma pool	6.03		
" + 1 ng cortisol	7.01	0.98	98
" + 2 ng cortisol	8.09	2.06	103
" + 3 ng cortisol	9.33	3.30	110
" + 4 ng cortisol	10.23	4.20	105

(c) Relationship of plasma volume to mass. Cortisol levels were assayed in various volumes of a fish plasma pool (September, seine-netted, male plasma) to determine whether the volume of the sample affected the result. A linear relationship between cortisol level and volume of plasma was obtained, again indicating high accuracy of the method (fig. 12).

(d) Precision. The precision within a batch of samples was determined by assaying 16, 0.1 ml. samples of pooled fish plasma. Precision between batches was determined by assaying a sample of pooled fish plasma as a standard with each batch of estimations, and comparing the results of 16 batches.

Within batches the concentration was 6.01 µg/100 ml. ± 0.67 (standard deviation) and between batches 5.88 µg/100 ml. ± 0.70 (standard deviation). These means were not significantly different ($p > 0.05$), coefficients of variation were 12.96% and 11.77% respectively.

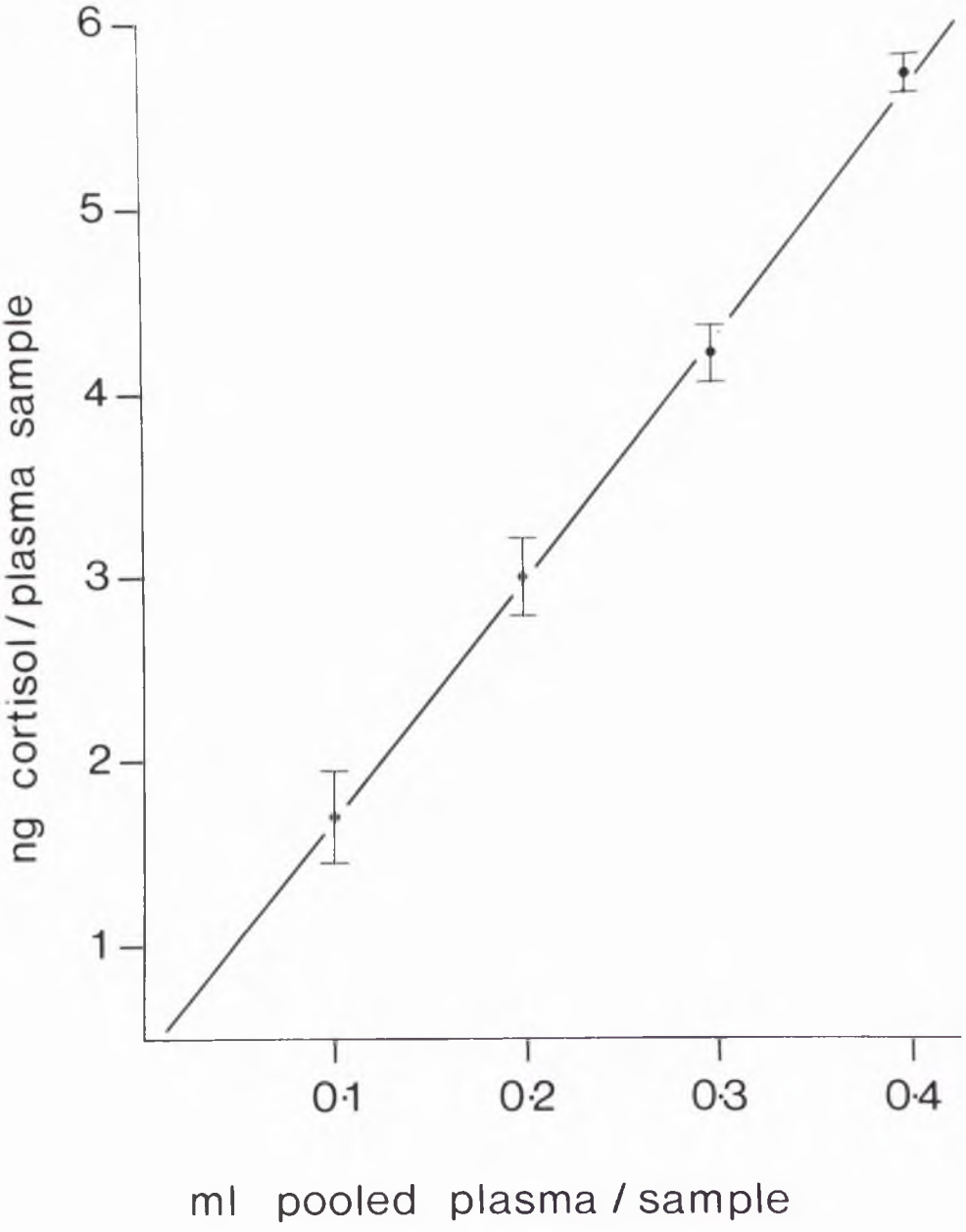


Fig.12. Accuracy, relationship of plasma volume to cortisol concentration.

(e) Specificity. Although competitive protein-binding is non-specific, specificity is attained by the inclusion of the chromatographic stage. Water blanks assayed with each batch consistently gave values which were not significantly different from zero, on the standard curve. Further investigation of specificity was by a comparison of results from competitive protein-binding with those from gas-liquid chromatography, the specificity of which had been extensively checked by physicochemical methods (Mason and Fraser, 1974). Twelve samples were analysed by both methods.

Samples were prepared for gas-liquid chromatography as follows: a neutral extract of plasma (5 ml.) containing (^{3}H)-cortisol (2500 cpm) was chromatographed on paper (B_5 system) and eluted. The residue was oxidised (Mason and Fraser, 1974) to androstenetrione which was again subjected to paper chromatography and eluted. Finally, the residue was esterified with heptafluorobutyric anhydride to form the 3-enyl heptafluorobutyrate of which suitable aliquots were injected into the gas-liquid chromatograph (p. 36-37).

Results from both methods determining the cortisol concentrations showed close correlation:

	no. samples	mean µg% cortisol	S.D.	range
GLC	12	6.75	2.27	3.8 - 9.2
CPB	12	6.70	2.20	4.1 - 9.0

The linear regression equation was $y = 0.97 + 0.28$ (95% confidence limits of the slope were 0.71 and 1.22) and the correlation coefficient (r) was 0.94 ($p < 0.001$).

Further specificity tests: The residue from paper chromatography of an extract of 80 ml. of fish plasma pool was physicochemically characterised by absorption of ultraviolet light and by formation of the phenylhydrazone derivative, characteristic of 17α,21-dihydroxylated-20-keto steroids (Porter and Silber, 1950; Nelson and Samuels, 1952). Parallel samples of pure cortisol were treated similarly. Plasma product, chromatographed cortisol and standard cortisol gave identical maximum absorption at 241 nm, indicating the presence of the Δ^4-3 ketone grouping. The phenylhydrazones of these three substances also gave identical maximum absorption at 360 nm indicating the presence of a cortisol type side-chain (fig. 2).

Absorption spectra were measured on a Beckman Model DB-GT spectrophotometer.

5. Discussion

An accurate, precise, sensitive and specific competitive protein binding method was established suitable for application to large numbers of small plasma samples from powan. The method is relatively simple and allows rapid analysis of samples, each batch requiring less than 2 days for completion.

C. RADIOIMMUNOASSAY OF POWAN PLASMA 11-DEOXYCORTICOSTERONE (DOC)

1. Introduction.

Preliminary investigations of powan plasma DOC levels by gas-liquid-chromatography (Table 2, p. 38) indicate low levels of only a few ng/100 ml. This amplifies the problems of assaying small volumes from individual fish, only pg quantities would be available for analysis and even using the most sensitive techniques currently available, pooled samples are necessary.

It is clear from the discussion of techniques available for steroid analysis that calorimetric and fluorimetric methods are of little value for this study. The disadvantages, for the present study, of the physicochemical methods, double isotope derivative assay and GLC with electron capture detection have been described also. Competitive protein binding analysis is not sufficiently sensitive for powan plasma DOC assay. Moreover, the application of this method by Brown and Strott (1971), in addition to requiring 10 ml. of plasma for each assay and then failing to detect levels in the lower half of the normal human range, requires two paper chromatographic purification stages making the technique unsatisfactory for analyses of large numbers of samples.

As previously stated, radioimmunoassay, by using a specific antibody to the steroid under examination, confers a considerable degree, although not absolute, of specificity on the method. Higher binding affinity between antibody and antigen than between CBG and steroid (as in the competitive protein binding assay) also results in very high levels of sensitivity making measurement at the pg level possible. The specificity of the assay means that only simple purification procedures are necessary making the method rapid and simple. The values obtained by radioimmunoassay of

DOC compare well with those obtained by specific but laborious and insensitive physicochemical methods (Arnold and Janes, 1971; Fraser et al., 1974).

It was decided to employ the technique of radioimmunoassay to study plasma DOC in the powan. Unfortunately, even using this sensitive approach, 2 ml. plasma samples were required and, hence, analyses were perforce conducted on pooled plasma samples rather than on samples from individual fish. An account follows of the method established and steps taken to demonstrate the validity of the method.

2. Materials.

Solvents. Borate buffer (pH 7.5, 0.1 M) contained 8.25 g. boric acid, 2.7 g. NaOH and 3.5 ml. conc. HCl in 1 l. water.

The B_3 chromatography system (Bush, 1961) contained petroleum ether (redistilled), benzene, methanol and water in proportions 13.2:6.8:4:1. All other solvents were as listed previously (p. 51).

Steroids. Unlabelled DOC (Sigma Ltd.) was stored in methanol at 4°C. Standard DOC solutions were prepared from this as follows and were also stored at 4°C, stock solution 100 µg/ml. (stable at 4°C, kept for several weeks).

1 ml. stock soln. up to 100 ml. MeOH ___ 1 µg/ml._____ (A)

1 ml. (A) up to 100 ml. MeOH _____ 1000 pg/0.1 ml.—(B)

Further dilutions were made to prepare standards of 6.25 - 100 pg/0.1 ml. methanol.

(^{3}H) DOC (New England Nuclear Corporation) of specific activity 40 Ci/mM was purified in the B_3 chromatography system before use and was repurified every 2 - 3 weeks; it was stored at 4°C in methanol.

For recovery purposes 25,000 cpm (37,500 dpm) equivalent to 139 pg DOC was added to each plasma sample. A 5000 cpm standard solution of (^{3}H) DOC was also stored at 4°C.

Materials for assay procedure. Bovine Y-globulin (Cohn fraction, approx. 99% Y, BG-11 Sigma) was dissolved in borate buffer at a 5% solution stored at 4°C.

DOC antiserum was provided by Dr. A. Wilson (M.R.C. Blood Pressure Unit, Glasgow). It was raised in rabbits using DOC 3-carboxy methyloxime (Wilson, 1973). 1:100 dilutions in borate buffer were separated into aliquots and stored at -20°C until required, when the aliquot was stored at 4°C.

For separating antibody-bound and free steroid in the assay procedure a solution containing 2.5 mg/ml. of charcoal and 2.5 mg/ml. of Dextran T80 (Pharmacia, Uppsala) in borate buffer was used and stored at 4°C.

All other details - washing of glassware, redistilling solvents and chromatography paper were as listed in the previous materials section (pp 51-52)

3. Method.

(a) Extraction of steroid from plasma. Pewan plasma (2 ml.) containing (^{3}H) DOC (25000 cpm in 0.1 ml. methanol) was extracted with 10 volumes of dichloromethane. After centrifugation (3 mins., 2000 rpm) the plasma layer was discarded.

(2 aliquots of the (^{3}H) DOC solution containing 25000 cpm/0.1 ml. were checked for tritium content).

A neutral extract was obtained with an alkali, acid, water wash (2 mls. each) as in the extraction for cortisol estimations (p. 53). The extract was evaporated to dryness (as for the previous technique for cortisol, all evaporations were under a stream of nitrogen gas).

A solvent partition stage included here, using 2 ml. hexane and 2 ml. 70% aqueous methanol, centrifuging and discarding the methanol layer, resulted in no improvement in the technique and was not therefore used in these studies.

The dry residue was transferred quantitatively to conical tubes by adding dichloromethane to the evaporated sample. The sample was again evaporated to dryness.

(b) Purification of extract. The residue was chromatographed on methanol-washed paper (Whatman 2) in the B_3 system (Bush, 1961) for 4 hours. The DOC region was located and eluted as described for cortisol (fig. 8). An aliquot of the eluate was assessed for (^{3}H) recovery and on this information duplicate aliquots containing 5000 cpm were taken for radioimmunoassay.

(Duplicate aliquots of the standard solution of (^{3}H) DOC (5000 cpm/0.1 ml.) were checked for tritium content,

0.1 ml. 5000 cpm standard = y cpm

" sample eluate = x cpm

$\therefore$ y x $\frac{0.1}{x}$ mls. sample eluate = y cpm

(taken for assay).

(c) **Assay.** 2 ml. glass tubes were used for the assay procedure. To each sample aliquot 0.1 ml. ethylene glycol was added. Duplicate standards were taken (0 - 200 pg containing 0.1 ml. ethylene glycol and 5000 cpm (^{3}H)-DOC). The contents of all tubes were evaporated to dryness in a vacuum oven at 35°C (when handling pg quantities of steroids higher recoveries are obtained after evaporating in the vacuum oven compared to using a stream of nitrogen as in the previous cortisol estimations). Evaporation is complete after about 30 mins. Antiserum solution of appropriate dilution (0.5 ml. containing 2% methanol and 0.5% bovine Y-globulin) was added to each tube and mixed with a vortex mixer for 10 seconds.

e.g. 20 mls. of a 1:6000 antiserum dilution contained:-

0.4 mls. methanol,

2.0 mls. Y-globulin (stored as 5% solution),

0.33 mls. antiserum (stored as 1:100 dilution),

17.27 mls. borate buffer.

The tubes were left at room temperature for at least 1 hour, and at 4°C overnight. The tubes were transferred to an ice-bath and the free steroid component was separated from the bound fraction by adding 0.5 ml. dextran/charcoal mixture. The tubes were mixed, centrifuged at 4°C, 10 mins, 25000 rpm and a 0.5 ml. aliquot from each tube was taken for

assessment of (^{3}H) content as in the previous method for cortisol estimations.

(d) Calculation of DOC content in plasma sample. The percentage of bound (^{3}H)-DOC in each of the standards,

$$100 \times \frac{\text{cpm} \times 2}{5000}$$ (1 ml. total incubation mixture, 0.5 ml. taken for counting)

was plotted against pg DOC (0 - 200 pg). In the same way percentage binding in the plasma sample was calculated and the pg content read from the standard curve (fig. 13).

DOC concentrations (ng/100 ml.)

$$= \text{pg} \times \frac{\text{cpm in recovery (25000 cpm) standard}}{\text{cpm in assay (5000 cpm) standard}} \times \frac{100}{2} \times \frac{1}{1000}$$

where a 2 ml. sample is used.

The validity of this method was assessed for reproducibility, sensitivity, specificity, accuracy and precision.

(4) Results of assessing validity of the radioimmunoassay method

(a) Standard curve.

Reproducibility, sensitivity and range of detection. Fig. 13 shows the reproducibility of 10 consecutive standard curves. The sensitivity (p.56) was 5 pg and the range of detection was from 0 to 200 pg. This represents 0 - 1000 pg per plasma sample or 0 - 50 ng/100 ml. if a 2 ml. sample is used (^{1}/5 of the extract is taken for assay).

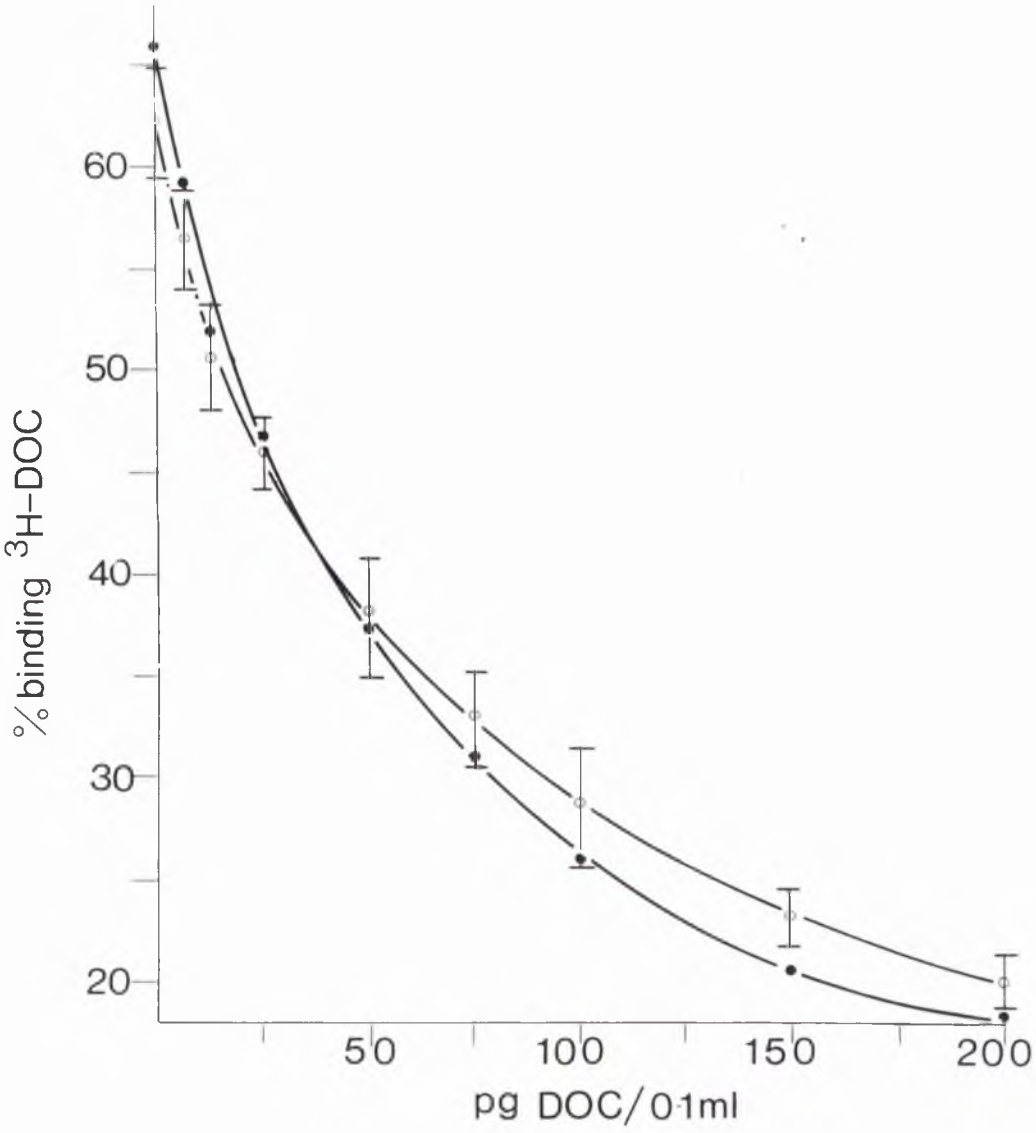


Fig.13. Reproducibility, mean ±1S.D. of 10 consecutive curves (∘—∘). Delayed addition of half gamma globulin (•---•).

Effects of varying amount of tritium and antibody dilution. Standard curves were compared varying the antibody dilution, using 4-5000 cpm (^{3}H)-DOC, and also varying the ratio of (^{3}H)-DOC:volume of antisera added. The optimum curve was obtained using the antisera at a dilution of 1:4000. 5000 cpm (^{3}H)-DOC was taken for the assay procedure and 0.5 ml. antiserum was added (Fig. 14).

(b) Recovery of (^{3}H)-DOC and accuracy. After the chromatography stage recovery of (^{3}H)-DOC was 85% ± 1.25 (S.D.), calculated from 20 samples. After the assay, the mean recovery of 20 samples was 73% ± 2.6 (S.D.).

Accuracy was checked by assaying samples of a human plasma pool containing known amounts of added DOC. Recoveries of added steroid ranged from 90 - 120% (Table 6).

(c) Precision. Ten, 2 ml. human plasma pool samples assayed within one batch gave a mean level of 8.5 ng/100 ml., the coefficient of variation was 9.66%. Ten samples assayed in different batches gave a mean level of 9.8 ng/100 ml. with a variation coefficient of 11.2%. There was no significant difference in results within and between batches ($p > 0.05$).

(d) Specificity. As already mentioned (p. 61) the use of an antibody, raised specifically to DOC, produces a technique of greater inherent specificity than, for example the competitive protein binding used for cortisol. Further specificity was achieved by chromatographic purification of the plasma extract in the Bush B_5 system. Previously (Fraser, Wilson and Holmes, 1973; Fraser et al., 1974) results from the technique described here have been compared with results of GLC estimations on the same human plasma samples. Results from the two techniques (reproduced by permission of M.R.C. Blood Pressure Research Unit, Western Infirmary, Glasgow) showed good correlation (Fig. 15) and as previously mentioned the GLC method is highly specific.

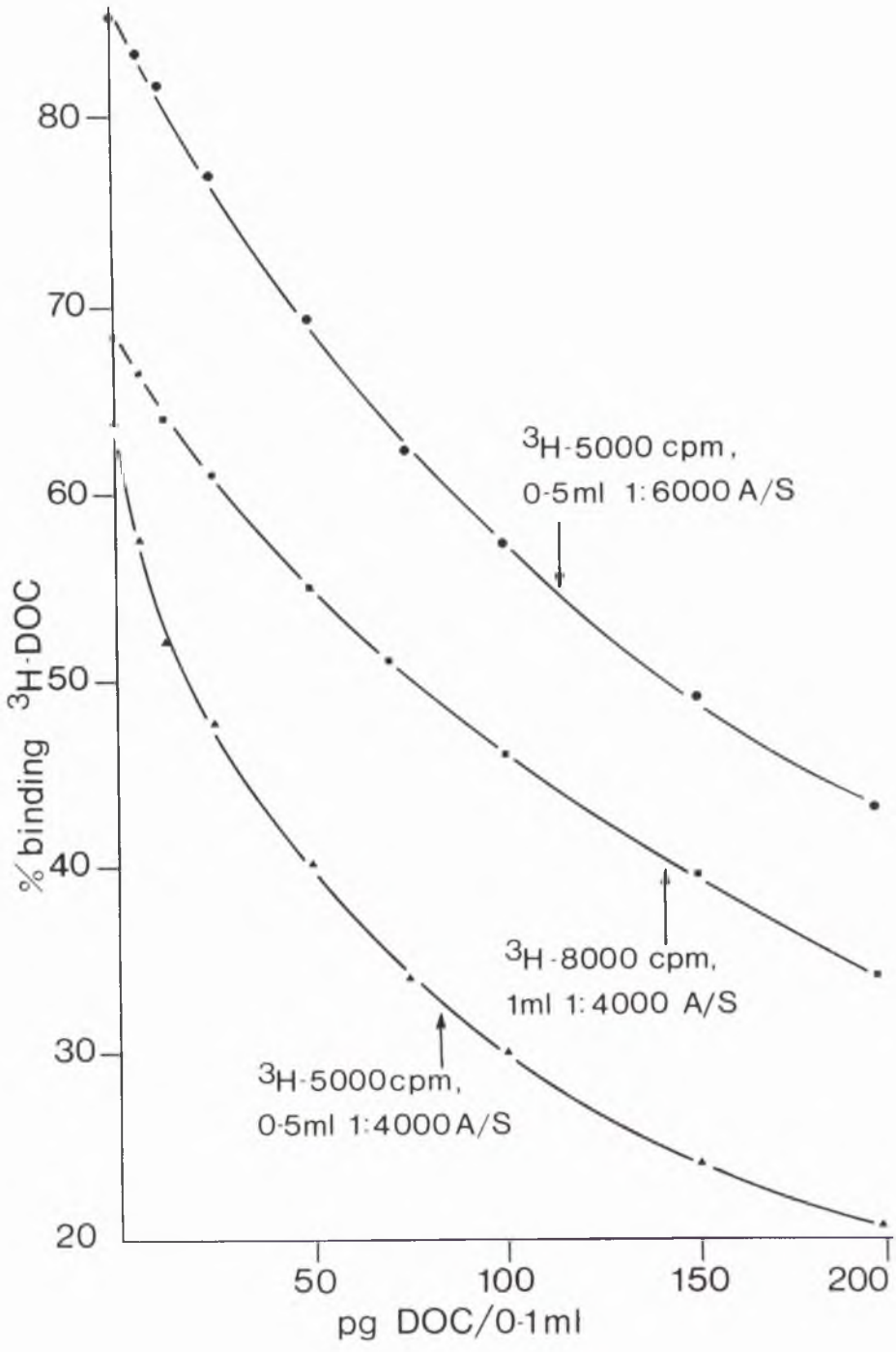


Fig.14. Effect of tritium and antiserum-A/S concentrations on standard curve.

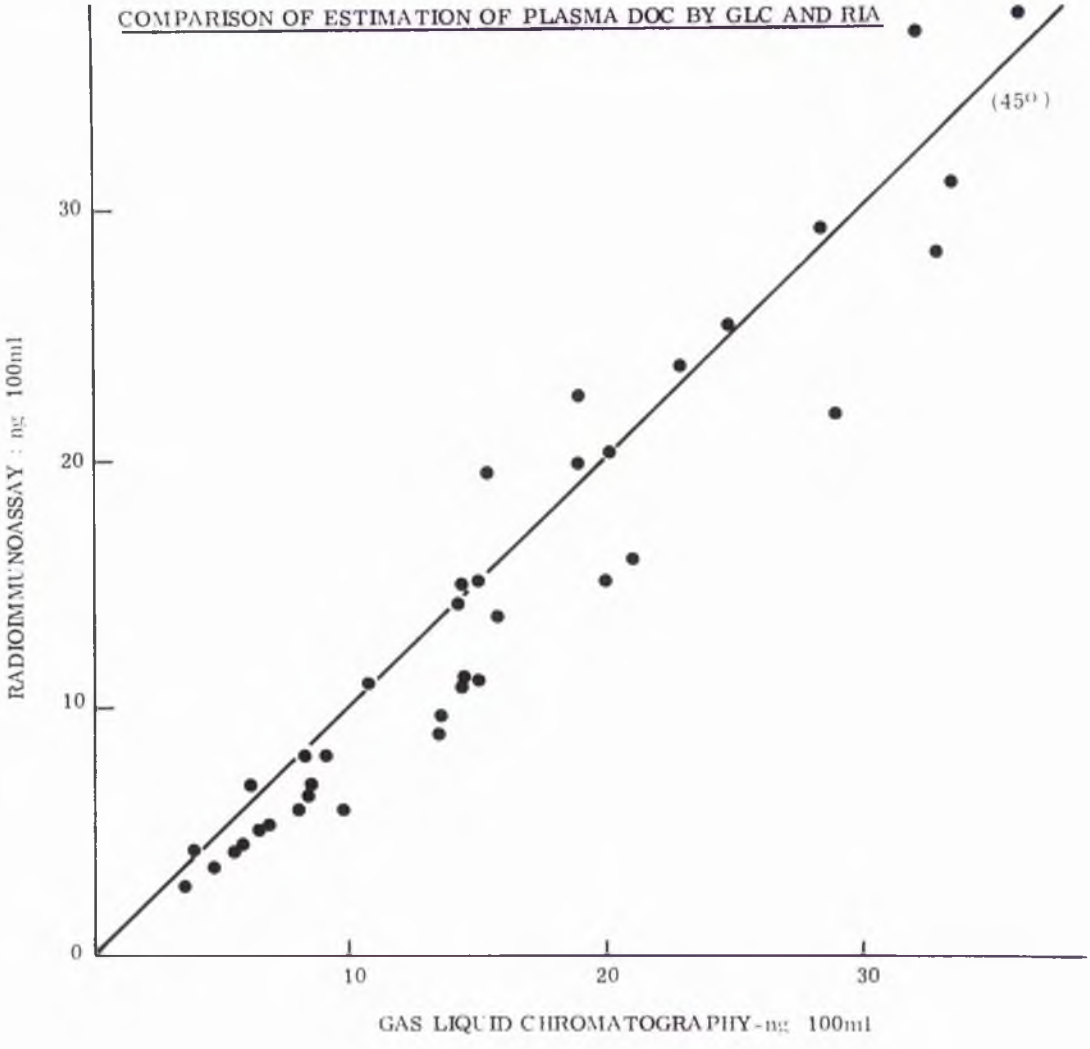


Fig.15.(courtesy of M.R.C. B.P.R.U.).

TABLE 6. Recoveries of added DOC.

Sample	Total DOC ng/100 ml.		% recovery added DOC
Human pool	8.0		
"	8.0	average	
"	6.0	7.2	
"	7.0		
"	7.0		
" + 2 ng/100 ml.	9.6		120
"	9.4		110
"	9.0		90
"	9.0		90
" + 5 ng/100 ml.	13.0		116
"	12.0		96
"	13.0		116
"	13.0		116
" + 10 ng/100 ml.	17.5		103
"	17.5		103
"	16.5		93
"	17.0		98

Further tests for specificity on the powan plasma are in progress, to compare results from this radioimmunoassay method with GLC measurements on the same pooled samples.

(e) Blanks. Initially, distilled water blanks indicated inconsistently high values of DOC content. Possible sources of interfering substances are solvents, such as water or those used in extraction and chromatographic procedures. It also seemed at least possible that the antiserum used might have been peculiarly susceptible to such contamination. Since high blank values have been a universal problem with steroid radioimmunoassay methods, the following investigations were conducted in an attempt to locate their source, or at least to reduce them to acceptable levels. These investigations were carried out on human plasma which was copiously available.

Components of extraction and chromatography checked. Renewing and checking all solvents and materials used in the extraction and chromatography stages had no effect on the high blank readings. Assays of samples from the B_3 system, methanol, paper eluate in methanol and (^{3}H)-DOC solution gave no indication of contamination at these stages.

Antiserum checked. For comparison, antisera from two other sources (C. Edwards, The Medical Unit, St. Bartholomew's Hospital, London and A. Rippon, Department of Chemical Pathology, St. Mary's Hospital, London) were used. Equally high blank values were obtained.

Keeping antiserum out of 4°C storage for the minimum time when taking aliquots for assays improved consistency of the standard curves but did not eliminate high blanks.

Effects of solubility. To determine whether solubility factors were affecting the blanks, all components taken for an assay procedure were doubled. Before the dextran-charcoal separation stage half the volume of each sample and standard were assessed for (^{3}H) content. The remaining half was counted after the separation stage, as usual. There was no indication of a solubility problem (Fig. 16).

Separation method. Ammonium sulphate precipitation (p. 46) was substituted for the dextran-charcoal separation. Standard curves obtained were less satisfactory and high blanks persisted.

Y-globulin protection. Since, in spite of high blank readings, consistent values were being obtained for plasma pool samples, with little variation, it seemed that the plasma was conferring some protective factor on the sample. Increased protein addition during the assay (p. 47) has previously eliminated high blank problems (Leyendecker, Wardlaw and Nocke, 1972). These workers found that dried down solvent residues affected the activity of binding proteins used in their method and increased Y-globulin added to each assay tube protected the proteins.

The DOC radioimmunoassay used in this study had previously been established (Wilson, 1973) using 0.5% Y-globulin. In the present study blank values were at first only eliminated using 1% Y-globulin - in particular when 0.5% was added as before with the antiserum, and 0.5% with the charcoal solution. Neither concentration altered the standard curve (fig. 13). With the increased quantities of carrier protein, blank values fell to between 0 and 6 pg which were not significantly different from zero point on the standard curve. However, after several preliminary assays, the amount of Y-globulin was reduced to 0.5% again and it was now found that blank readings remained indistinguishable from zero. There were

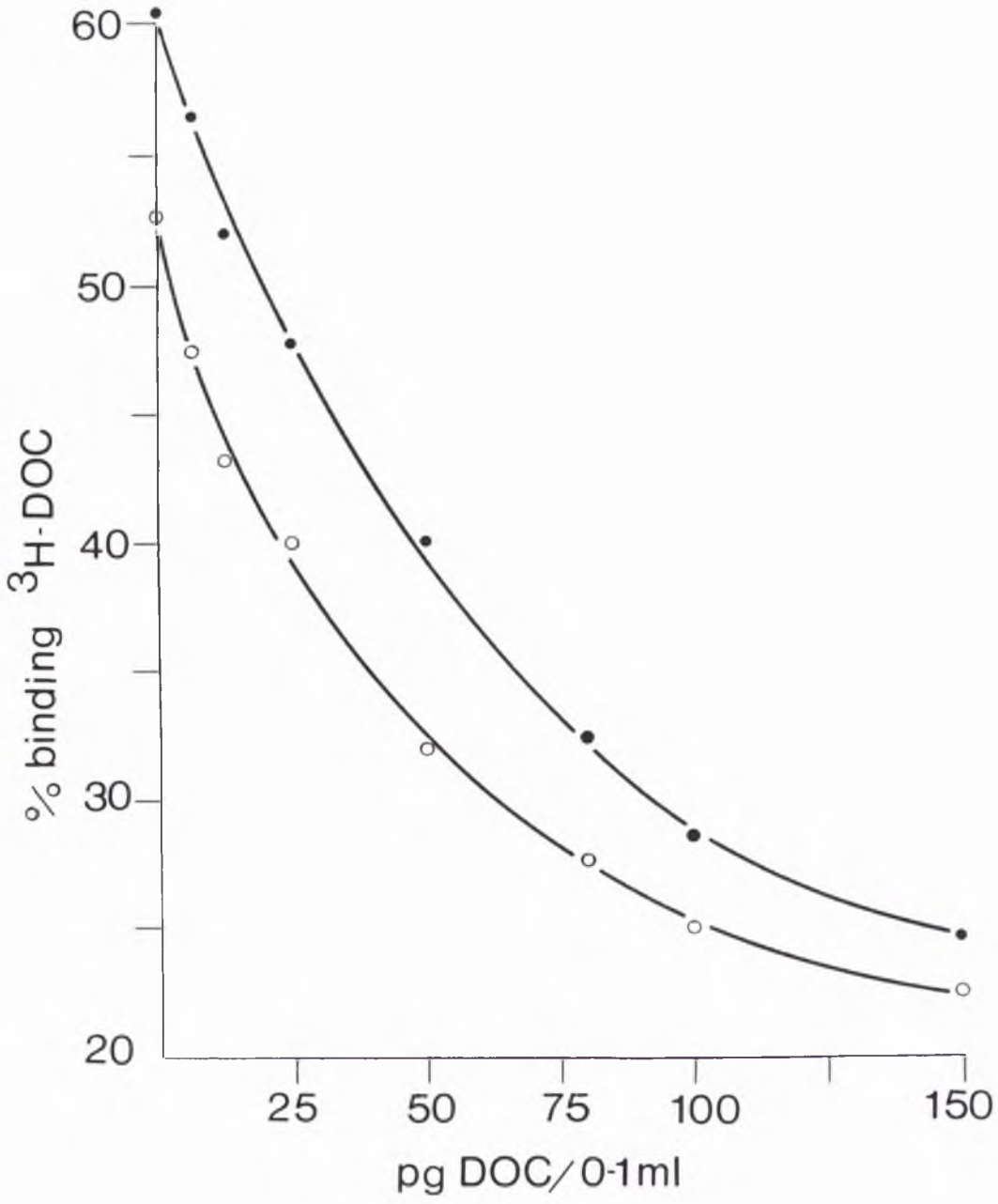


Fig.16. Standard curve,
before solubility correction ○—○ ,
after solubility correction •—• .

no explanations for blank values reverting to zero at this stage. However, all future blank values maintained these zero levels.

It was decided to include, with each batch of 18 powan samples, 2 samples of water and also 2 samples of plasma from humans suffering from primary adrenocortical insufficiency (Addison's disease). The latter produced results, as expected, of 0 - 0.5 ng DOC/100 ml. in all samples (indistinguishable from zero on the standard curve).

5. Discussion

Assessment of the radioimmunoassay method indicated an accurate, precise technique with high sensitivity for determining powan plasma DOC levels. Initial blank values are difficult to comment on. It seems unlikely that lack of skill on the part of the investigator would provide an explanation since precision and accuracy were high even before the high blanks were eliminated, and there had been no indication of contamination from the materials used. Initially the increased carrier protein concentration eliminated high blanks though this precaution was no longer necessary after several trial assays had been completed. In spite of a lack of interpretation of the cause of these high blanks in the development of the method, throughout all assays on the powan samples listed in this study, blank values were indistinguishable from zero.

Proof of specificity of the radioimmunoassay for DOC presents more problems than that of the previous competitive protein binding method for cortisol. This is because small (pg) quantities of steroid are being handled in the DOC tests, compared to cortisol which is present in the plasma in much higher concentrations. Therefore, the vast amount of

plasma required for stringent specificity tests imposes restrictions on the checks which can be made for the DOC radioimmunoassay. However, as described (p. 61), radioimmunoassay is itself a specific-mass measurement with further specificity achieved, in this study, by inclusion of a chromatographic stage. Results from measurements on powan plasma by both GLC and radioimmunoassay should complete the confirmation of specificity of the radioimmunoassay method.

The two methods established in this section of the study enable measurement of cortisol and DOC in powan plasma. The following chapter describes the application of the techniques with results of the levels of these steroids in the powan.

CHAPTER 5.

RESULTS OF PLASMA CORTISOL AND DOC MEASUREMENTS IN THE POHAR

A. INTRODUCTION

Previous identifications of corticosteroids in teleosts (from *in vitro* and *in vivo* studies) have been discussed (pp. 31 - 35). Identification of cortisol and DOC specifically, are reviewed in more detail below, and their physiological roles are discussed.

1. Cortisol

(a) Previous techniques used for estimating teleost plasma cortisol levels. The relative merits of different techniques available for measuring steroids have already been discussed (pp. 42-48). Techniques used for measuring cortisol levels in teleosts have been extensively reviewed (Idler, 1972) and will be only briefly discussed here.

The first isolation of cortisol in teleost plasma was from *Cyprinus carpio*, the carp and the flounder *Pseudopleuronectes americanus* (Bondy, Upton and Pickford, 1957). Since this first identification many studies have been made, though on very few of the many teleost species. Also, measurements have often been of total 17,21-dihydroxy-20-ketosteroids (17OHCS) rather than of cortisol alone (though there is no evidence of a new, distinctive teleost steroid that might be included in this group).

Techniques used for measuring the cortisol levels have included the relatively non-specific, insensitive methods of U.V. absorption spectra analysis and formation of chromogens by oxidation or with tetrazolium dyes (Bondy, Upton and Pickford, 1957; Phillips and Chester Jones, 1957; Nandi and Bern, 1960). Fluorimetric methods using acid or alkali

fluorescence have been employed often as a means of identifying and measuring cortisol (Phillips, 1959; Chester Jones, Phillips and Holmes, 1959; Donaldson and Fagerlund, 1968; Fagerlund and Donaldson, 1969). The most common method used has been the Porter-Silber chromogen test (Hatey, 1954; Robertson et al., 1961; Leloup-Hatey, 1964a, b; Schmidt and Idler, 1962). It has been shown that lactic acid under certain extraction conditions produces a typical Porter-Silber reaction, also that fish engaging in extreme exercise produce great quantities of lactic acid (Black, 1957). This fact may have resulted in spurious cortisol levels in some cases, but recently, modified and frequently used extraction techniques overcome this problem by removing lactic acid. Competitive protein binding has been used for measuring cortisol in only a few cases (Bradshaw and Fontaine-Bertrand, 1968; Ball et al., 1970).

(b) Postulated physiological roles of cortisol. Just as it is necessary to validate the biochemical technique used for assaying plasma samples, it is equally necessary to establish the biological validity of the work. Most investigations so far have been designed to establish the relationship of cortisol to certain physiological processes and this applies even to the most extensively studied teleosts, the Salmo and Anguilla species. Measurements have therefore often been made at times corresponding to extreme physiological states such as spawning and migration. No study has been carried out covering all stages of maturity in the complete reproductive cycle of a species, over large numbers of individuals and taking into account the effect of catching and handling methods on the cortisol levels. Consequently, it is difficult to define 'normal' cortisol levels in teleost plasma. Furthermore, without sufficient evidence of secretory rates, metabolic clearance rates and of target organs it is difficult to establish the physiological role of the steroid. Previous evidence has suggested the following functions of cortisol.

Osmoregulation, migration and motor activity. It has been suggested that corticosteroids, particularly cortisol, are involved in osmoregulation. Changes in adrenocorticotropic cells of the pituitary, and in adrenocortical cells, corresponding to osmoregulatory changes, have been observed (Pickford and Ats, 1957; Olivereau, 1962). Removal of adrenocortical tissue, preceeding electolyte measurements has been achieved in Anguilla anguilla (Chan et al., 1967; Chan, Rankin and Chester Jones, 1969) and resulted in a decline in plasma electrolytes, urine production and inulin clearance rate. Cortisol administered (20 µg/100 g. day) to these fish maintained normal electrolyte balance. However, lack of information on normal hormone levels in teleosts presents problems of distinguishing between pharmacologically high and physiological doses of cortisol in these experiments which may lead to misinterpretation of results. Recent work on Anguilla anguilla (Ball et al., 1970) involving osmotic adjustments of eels transferred between salt- and fresh-water showed that the only change in plasma cortisol level was a transitory increase during the first few days after transfer from fresh- to salt-water. It was suggested that cortisol may be necessary for triggering osmoregulatory mechanisms vital for existence in salt-water. Salmon smolts have been shown to have high cortisol levels (up to 135 µg/100 ml.) compared to other stages in the life-cycle (Fontaine and Hatey, 1954; Leloup-Hatey, 1964b).

A complication characteristic of teleost studies is that elevated cortisol levels may be due to increased motor activity rather than to changes in osmoregulatory systems themselves. For instance, anaesthesia of Cyprinus carpio, before transfer from fresh- to salt-water prevented rise in 17 OHCS level which otherwise occurred (Leloup-Hatey, 1964a). Forced swimming in Salmo gairdneri caused increases in 17 OHCS levels (Leloup-Hatey, 1964a) but further work contradicted these results (Hill

and Fromm, 1968). Schmidt and Idler (1962) found high plasma cortisol levels in Oncorhynchus nerka, taken from turbulent water, where strenuous motor activity was occurring.

In conclusion, it seems that cortisol levels may rise at times of prolonged or vigorous exercise and that cortisol may be involved in osmoregulatory changes. Whether the involvement of cortisol is direct or through indirect mechanisms such as glycogenesis or glycogenolysis is unknown.

Reproduction, maturation and spawning. Salmonids are the only teleost group in which cortisol levels have been measured over the reproductive cycle but results are incomplete, conflicting and difficult to interpret. In Salmo salar, the Atlantic salmon, no elevation of cortisol levels were found in either maturing or spawned fish (Fontaine and Hatey, 1954; Leloup-Hatey, 1964). In Oncorhynchus kisutch high 17 OHCS levels were found in plasma from maturing, spawning and spent fish (Hane and Robertson, 1959; Robertson et al., 1961) and hyperplasia of adrenocortical tissue was also found in mature and spawning fish (Robertson and Wexler, 1959).

During the spawning migration of the salmon, the physiological state of the fish resembles that of Cushing's syndrome in man (Robertson et al., 1961) and it was suggested that the accompanying increase in activity of adrenocortical cells was the cause of high cortisol levels in these fish, but increased motor activity in migration, or impaired clearance of steroids, or effects of catching methods could all be the cause of the high cortisol levels identified not necessarily the physiological state resembling Cushing's syndrome. Hence, it is clear that the role of cortisol or other corticosteroids in the reproductive cycle of teleosts is unsolved.

Diel changes in cortisol level. It is still unknown whether teleosts, like mammals, exhibit a rhythmic pattern of cortisol secretion. It is difficult to apply controlled conditions to fish to ensure that all other external factors do not influence results. The only evidence so far is on Ictalurus punctatus, the channel catfish (Boehlke et al., 1966) in which total glucocorticoids are reported to rise in mid- afternoon. However, experimental conditions are not described nor are specificity tests reported.

In the absence of firm evidence of a diel pattern of corticosteroid secretion, considerable care must be taken both in interpreting random assays of fish steroid levels and also in the design of studies of adrenocortical function. An obvious approach, adhered to in the studies described in this project, is to standardise, as far as possible, the times at which plasma samples are taken.

Effect on circulating blood cells. ACTH and cortisol may both be involved in the short-term response of white blood cells to stress (Ball and Slicher, 1962; Slicher, Pickford and Ball, 1962). In Fundulus heteroclitus and Poecilia latipinna hypophysectomy decreases the number of circulating white blood cells. Administration of ACTH and cortisol can maintain normal white blood cell count in these fish. Also, cold stress in these species results in initial leukopenia followed by leukocytosis and hypophysectomy abolishes the leukocytosis.

Protein and carbohydrate metabolism. Experiments involving hypophysectomy, adrenalectomy, administration of corticosteroids and use of corticosteroid synthesis blockers indicate that, as in other vertebrates, cortisol promotes glycogenesis and glycogenolysis. This subject has been well reviewed by Chester Jones et al. (1969)

Stress of captivity and catching methods. The effect of stress on teleost corticosteroid levels is important. Most evidence has been from the salmon species (Hane et al., 1966; Fagerlund, 1967). Hane et al. found elevated cortisol levels in fish bled in the period from 3 to 48 hr. after landing the nets and they attributed these elevations to restraint in confinement rather than due to the netting process. Elevated cortisol levels were not due to moribund condition as the mortality rate was not high. Fagerlund (1967) found that anaesthetising salmon for transport (using 2-phenoxyethanol) still resulted in high cortisol levels in some instances, also the females showed more cases of elevated cortisol levels.

Social behaviour. It has been suggested that the adrenocortical tissue and the steroids it secretes are involved during social stress in teleosts. This work is based only on histological evidence, where adrenocortical cell changes were observed (Fuller, unpublished) and from these results it is suggested that in Xiphophorus helleri, the swordtail, corticosteroid secretion may be correlated with the position of fish in social hierarchies. Similar reports are from Greenberg (1947) studying Lepomis cyanellus, the green sunfish.

2. DOC.

(a), Previous techniques used for estimating teleost DOC levels. Even fewer studies have been carried out on DOC in teleosts. There is only one report (p. 32) of DOC production from in vitro work where endogenous precursors were used (Sundararaj and Goswami, 1969). Identification methods used in their study can only be considered tentative as they were based on chromatographic behaviour, chemical reactions of the steroid (colour tests) and U.V. absorption spectra, however, they still provide the first evidence of endogenous DOC.

Production of DOC from exogenous precursors in head-kidney incubates has been obtained more frequently (p. 32). The results of Arai, Tajima and Tamaoki (1969) working on Salmo gairdnarii showed that whilst DOC was formed from progesterone as precursor it was not formed from pregnenolone. It was suggested (Idler, 1972) that this may have been due to the fact that 17α-hydroxylation of progesterone (fig. 2) is less efficient, relative to C-21 hydroxylation, than 17α-hydroxylation of pregnenolone. For this reason it was also suggested that, in the presence of a strong 17α-hydroxylase in teleosts, the accumulation of DOC might not occur for secretion by the adrenocortical cells. However, another report (Whitehouse and Vinson, 1973) has shown that DOC is produced in vitro, in pewan head-kidney incubates, from exogenous pregnenolone.

Reliable identification of DOC in teleost plasma is lacking. A double isotope dilution technique was used to measure DOC in one serum pool from Carassius auratus (Chavin and Singley, 1972), but identification was not positive (p. 34). The result was 800 ng/100 ml. serum which compared to human DOC levels (2.8 - 16 ng/100 ml.) is exceedingly high, also levels of other steroids found in the same study were higher than levels found in other studies in vertebrates. For instance, the aldosterone level was 110 ng/100 ml. (normal human levels are 4 - 18 ng/100 ml.). Even taking into account the high proportion of female serum in the pool, which the authors suggest might account for the high corticosteroid levels, the levels are still very high. The authors do not describe the catching technique employed for obtaining the fish, also the fish were killed only 3 - 4 days post- apture which suggests that high DOC levels may have been due to effects of methods of catching and handling if these factors affect DOC as they do cortisol levels (p. 78). The same report of Chavin and Singley, describes identification of plasma DOC in Oncorhynchus nerka by

Idler (1962), but it is in fact 20β-dihydrocortisone, not DOC, that was found in this latter study.

(b) Postulated physiological roles of DOC. There is little evidence for the possible function of DOC in teleosts. There is no evidence that DOC possesses, as in mammals, a mineralocorticoid function. An unusual function has been ascribed to DOC (Goswami and Sundararaj, 1971a, b), namely that in the Indian catfish, Heteropneustes fossilis, DOC is involved in maturation and ovulation of oocytes. The results from this report are summarised below:

(i) In vitro culture of ovaries indicated that exogenous cortisol and DOC, or their respective acetates were all potent maturation-inducing agents. Luteinising hormone was comparatively less effective. All other hormones used were ineffective.

(ii) Fish injected with LH or DOC acetate induced oocyte maturation, the effects of LH occurred 2 hours after that of DOC.

(iii) Ovarian pieces cultured in vitro from 1 to 8 hours, after LH or DOC acetate injections, completed maturation in vitro if taken from fish 2 hrs after DOC acetate injection but only after 4 hours following LH injection.

The conclusion was that the terminal hormones acting on the catfish oocytes to induce maturation and ovulation are the corticosteroids. The time lag of LH in (ii) and (iii) was considered to be the time, in vivo, for LH to act on the adrenocortical tissue to elicit corticosteroid secretion. LH added to head-kidney incubates increased the ratio of DOC: cortisol produced, in favour of DOC. It is possible that, in vivo, LH is secreted at ovulation time and increases the DOC level thus affecting the DOC: cortisol ratio, and perhaps if ACTH secretion is increased the ratio may

be changed in favour of cortisol which may bring about atresia (rather than ovulation) under stressful conditions (Sundararaj, per. comm.).

Aim of this section of the project

Direct investigation of the relationship between LH and DOC in individual fish is at present precluded by lack of methods for estimating these hormones in teleosts. One aim of the present study was to remedy this situation with respect to DOC. Before meaningful measurements can be made on plasma cortisol and DOC in teleosts, it is essential to investigate the effects of the catching and handling methods used on the fish, which might have varied effects on steroids in different species. Such investigations could avoid misinterpretation of the significance of steroid levels identified.

The biochemical techniques for measuring pewan plasma cortisol and DOC have been described in the previous chapter. This section deals with the levels of cortisol and DOC identified by these techniques. The aim was to study the effects on the steroid levels of,

(i) catching and killing methods,

(ii) aquarium maintenance,

(iii) post-mortem delay before blood extraction,

and in the light of this background information to study,

(iv) seasonal variations in cortisol and DOC levels.

In this fresh-water species osmoregulatory and migration problems are eliminated.

B. EXPERIMENTS AND RESULTS

For these studies, immature powan (in their first year), and abnormal individuals were discarded, except where stated otherwise. Fishing methods, bleeding of fish and storage of plasma have been described earlier (pp. 8-9) and the methods of steroid estimations have been described in Chapter 4.

As cortisol measurements were on 0.1 ml. plasma samples from individual fish, and DOC measurements were on 2 ml. samples (p. 62) the levels of the two steroids presented here do not represent levels in the same fish for both steroids. Although, in most experiments, fewer samples were analysed for DOC content than for cortisol, each DOC sample represents the mean level of DOC in 5 - 10 fish. Individual variation in the DOC levels would therefore be reduced.

Experiment 1: The effect of catching and killing methods.

The aim of this experiment was firstly, to compare plasma cortisol and DOC levels in fish caught by gill-netting with levels in fish caught by seine-netting. Secondly, levels were compared in fish killed by the quick and convenient method of concussion with those steroid levels of fish anaesthetised with a lethal dose of MS222 (Sandos). In the latter case, powan were placed in tubs of loch water containing a lethal dose of MS222 (approx. 5 ppm), immediately after removal from the net.

Cortisol. For cortisol analysis, fish were collected in September. 40 were gill-netted, and of these 20 (10 ♂, 10 ♀) were killed by concussion and 20 (10 ♂, 10♀) by MS222. 40 fish caught by seine-netting were treated likewise.

Whichever killing method was used, gill-netted fish had significantly higher levels than seine-netted fish (Tables 7, 8) though anaesthetised

seine-netted fish had significantly lower levels than concussed specimens ($p < 0.001$), MS222 did not produce lower levels in gill-netted fish. These results also showed that male and female cortisol levels were not significantly different ($p > 0.4$).

DOC. DOC levels were also measured in fish collected in September (Table 9). No significant differences in male and female levels were found ($p > 0.05$). Again, steroid levels in gill-netted fish were consistently higher than in seine-netted fish ($p < 0.001$, for male and female comparisons of both netting methods). Though only one pooled sample was available for the study of anaesthesia as a killing technique the DOC level was not lower than the mean level in concussed fish.

In summary, gill-netting caused elevation of both cortisol and DOC levels in male and female powan. MS222 only lowered cortisol levels in seine-netted fish, the effect on DOC is uncertain as more samples are needed for statistical analysis. DOC and cortisol levels do not differ significantly in males and females.

Experiment 2a. The effect of aquarium maintenance.

All specimens were mature fish caught in seine or gill-nets and were selected as being the most likely individuals to survive at the time of capture. They were transferred from the nets to polythene tanks 1 m. by 1 m. by 0.5 m. containing loch water, and were killed by concussion 1 to 3½ days later.

Cortisol. (Table 10). Aquarium maintenance resulted in a fifteen-fold increase in the only seine-netted specimen. Gill-netted females showed a two-fold increase in cortisol levels. Gill-netted males also showed a significant ($p < 0.01$) rise in cortisol levels compared to males bled immediately after catching, caught by the same method at the same time of year.

TABLE 7: Plasma cortisol levels (µg/100 ml.) of Coregonus lavaretus caught and killed by A, gill-net and concussion; B, gill-net and MS222; C, seine-net and concussion; D, seine-net and MS222. Fish caught in September.

	A		B		C		D	
	♂	♀	♂	♀	♂	♀	♂	♀
	11.34	8.10	11.9	18.5	9.45	2.70	1.12	4.96
	10.26	27.34	15.1	12.3	4.59	2.70	1.68	1.12
	30.20	24.84	16.9	20.0	2.70	1.19	0.56	0.28
	8.10	14.04	14.2	18.2	1.94	1.62	0.56	1.12
	6.72	25.22	14.7	16.8	7.56	0.56	2.76	1.68
	15.36	22.56	15.5	17.6	6.72	3.36	0.53	2.52
	18.24	21.60	15.8	19.1	8.40	4.48	1.05	0.53
	20.16	16.32	11.8	17.8	4.48	2.74	2.65	0.27
	12.48	24.00	17.2	19.5	1.68	4.94	0.80	0.53
	21.12	21.12	14.9	24.0	3.92	5.20	2.52	0.53
Mean	15.40	20.51	14.8	18.4	5.14	2.95	1.42	1.35
Standard deviation	6.85	5.86	1.8	2.9	3.30	1.57	0.91	1.42

TABLE 8: Statistical analysis of effects of catching and killing methods on plasma cortisol concentrations. Significance was accepted at the 1% level.

Groups (Table)	Comparison	p
A v. B.	concussion v. MS222, gill-netting.	>0.5
C v. D	concussion v. MS222, seine-netting	<0.001
A v. C	gill-netting v. seine-netting concussion	<0.001
B v. D	gill-netting v. seine-netting MS222	<0.001
♂ v. ♀	sex difference	>0.4

TABLE 9: Plasma DOC levels (ng/100 ml.) of Gerreomorpha lacuratum, mature fish caught in September. Each sample represents the level in a pooled sample from 5 - 10 fish.

	♂	♀
A. Gill-netted, killed by concussion	7.5 8.6 6.0 6.5 4.0 5.0	8.6 9.5 5.5 6.5 7.0 4.5
B. Seine-netted, killed by concussion	3.4 3.0 2.5 2.2 1.4	3.5 2.8 2.8 3.6 2.4
C. Seine-netted, killed by MS222	2.9	2.7

TABLE 10: Plasma cortisol levels (µg/100 ml.) of *Coregonus lavaretus* maintained in aquaria.

Catching method	Month	Mean cortisol level of fish caught in same month, killed by concussion	Hours in aquarium	Sex	cortisol level
Seine	September	3.7	55	♂	53.9
Gill	February	10.1	24	♂	8.1
"	"	"	"	♂	10.2
"	"	"	"	♂	17.7
"	"	"	"	♂	18.7
"	"	"	48	♂	17.9
"	"	"	"	♂	24.8
"	"	"	"	♂	21.2
"	"	"	"	♂	26.4
"	"	"	"	♂	17.3
"	"	"	80	♂	17.0
"	"	12.2	48	♀	33.0
"	"	"	"	♀	28.1

DOC. A pooled sample of plasma from aquarium maintained females had a DOC level of 9.6 ng/100 ml., and from males, two pooled samples showed a mean DOC level of 7.3 ng/100 ml. These levels were comparable to DOC levels in gill-netted fish but were significantly higher than levels in seine-netted fish.

In conclusion, aquarium maintenance of gill-netted and seine-netted powan for periods of 1 to 3½ days causes elevation of cortisol and DOC levels.

Experiment 3: The effect of post-mortem delay before blood extraction.

Powan caught by seine-netting in May were killed by concussion. They were bled after 0, 2, 4, 8 and 16 hours to investigate the effect of post-mortem delay before bleeding specimens. For the fish bled immediately (0 hrs), bleeding, centrifugation, separation and storage of plasma were carried out in the boat so that the maximum time between catching and blood sampling was ten minutes.

Cortisol. Plasma cortisol levels decreased with time elapsed since killing, markedly after 2 hours (fig. 17). Predictably, bleeding became increasingly difficult, as time passed, in the latter groups.

DOC. As mentioned above, bleeding was difficult in fish kept for longer than 4 hours, consequently collection of 2 ml. pooled samples for analysis of DOC content was particularly difficult. Results show no corresponding drop in DOC levels after 2 hours, as with cortisol levels, but after 4 hours DOC levels also fall off markedly (fig. 18).

These results emphasise the importance of bleeding fish as soon as possible after catching. For all routine sampling in this study, fish were bled within one hour of catching.

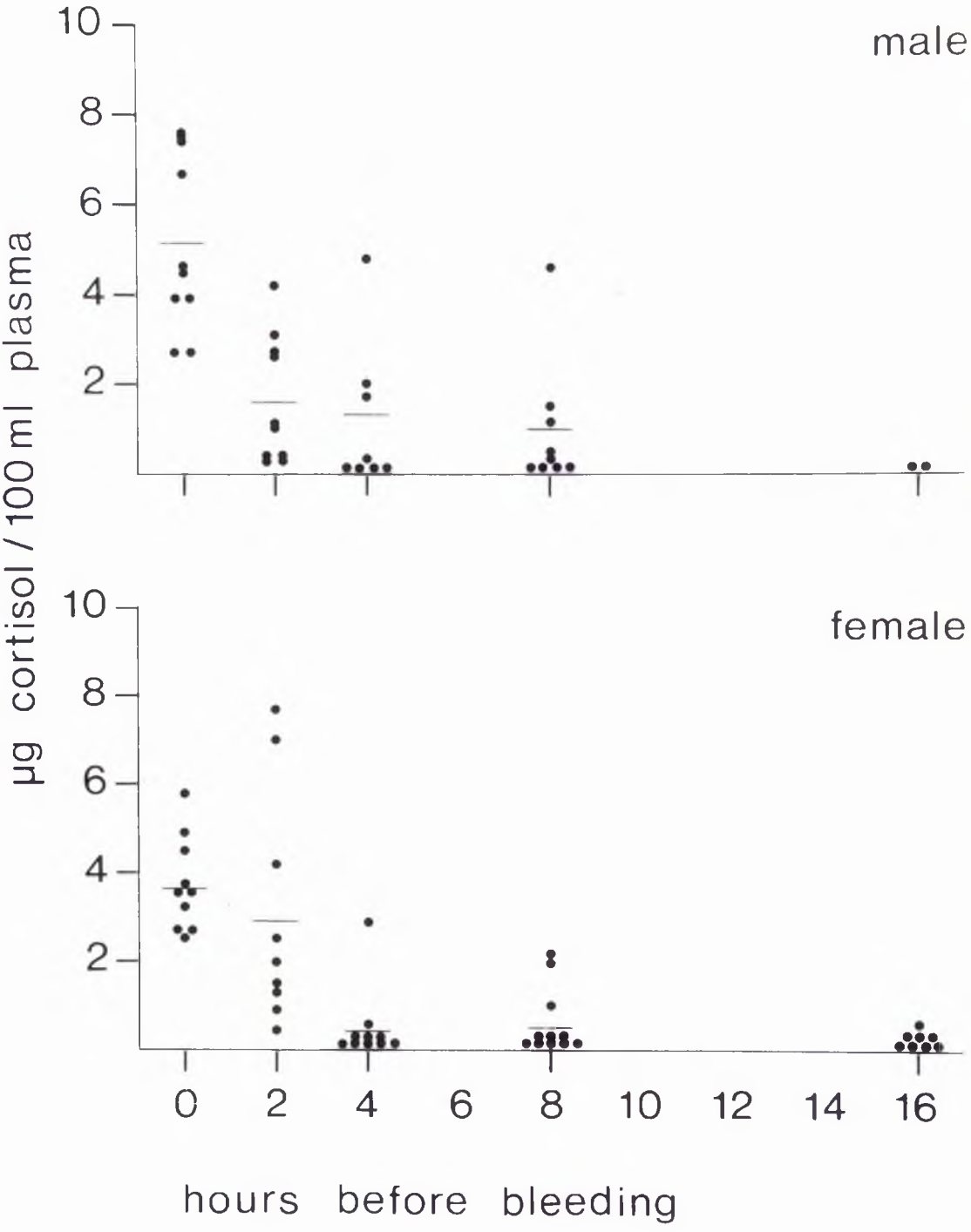


Fig.17. Effect on cortisol levels of delay before bleeding, mean values (—), individual values (•).

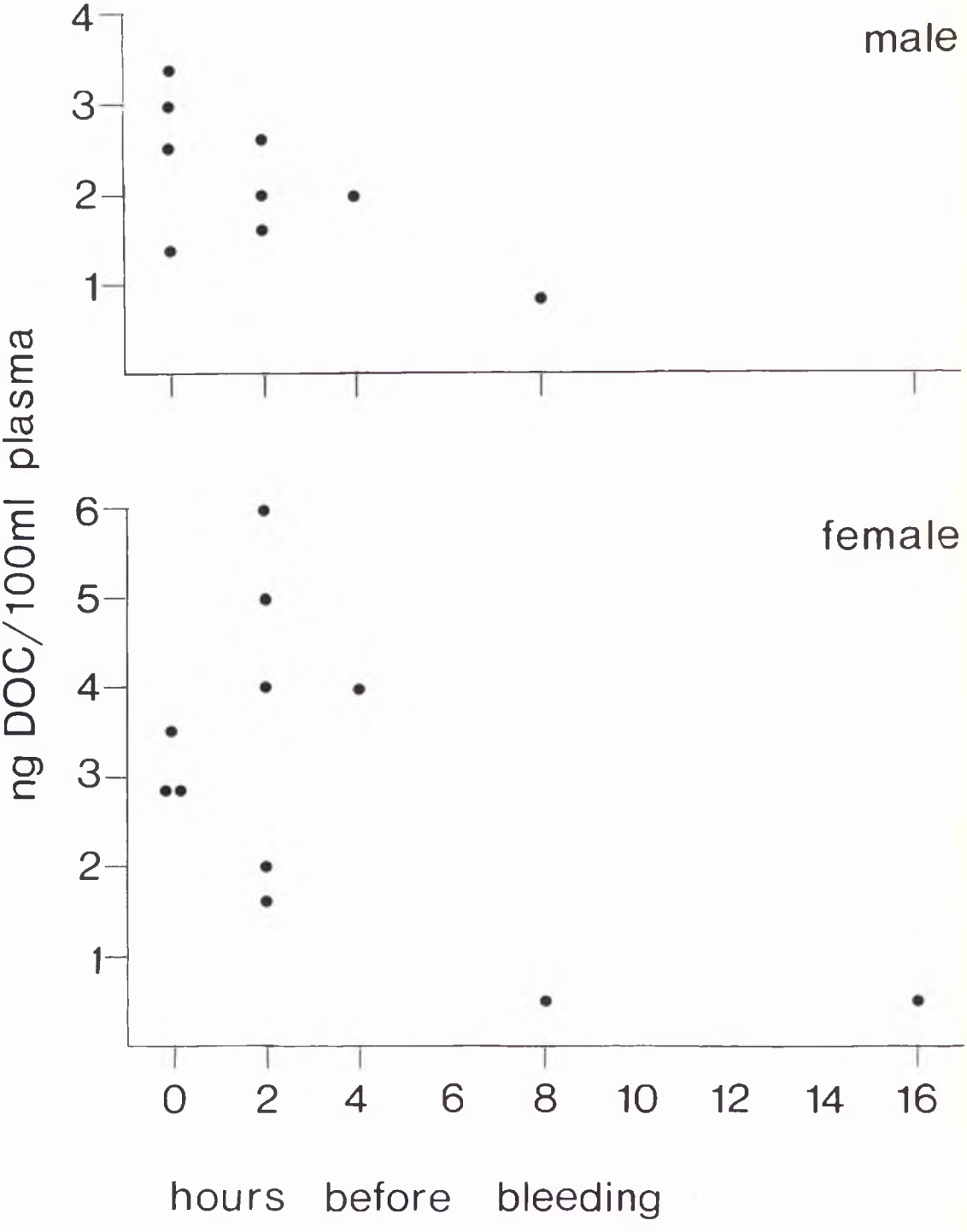


Fig.18. Effect on DOC levels of delay before bleeding.

Experiment 4. The effect of length of time spent in the gill-net.

Gill-nets were set in open water (not on the spawning grounds) in January, for periods of 3, 6, 18, 24 48 and 72 hours. For each time period investigated blood was pooled from groups of ten fish keeping male and female plasma separate. Plasma was pooled in order to obtain mean values of steroids from fish in the same net, as fish would obviously be entering the nets throughout the period for which it was set. The aim of this study was to investigate the results of laying nets for different durations before standardising a length of time with least effect on corticosteroid levels for routine sampling.

Cortisol and DOC. Little change in cortisol (fig. 19) and DOC (fig. 20) levels occur in the first 18 hours in gill-nets but there is an increase thereafter.

There may be a greater rise in cortisol and DOC, with time, than is immediately apparent from the results, as fish will be entering the nets at all times. The long-term nets will therefore contain a proportion of newly caught fish. The increase in standard deviations in the long-term nets confirms this, consequently the mean value would probably be higher if no newly caught fish were present.

For routine-sampling, gill-nets were laid for not more than 18 hours.

5. The effect of the annual reproductive cycle on cortisol and DOC levels.

Powan were collected by gill-netting, and were killed by concussion throughout the year. When possible, seine-netting was also carried out (see Discussion). Seasonal variations in cortisol and DOC levels were correlated with the annual reproductive cycle (pp. 13-15).

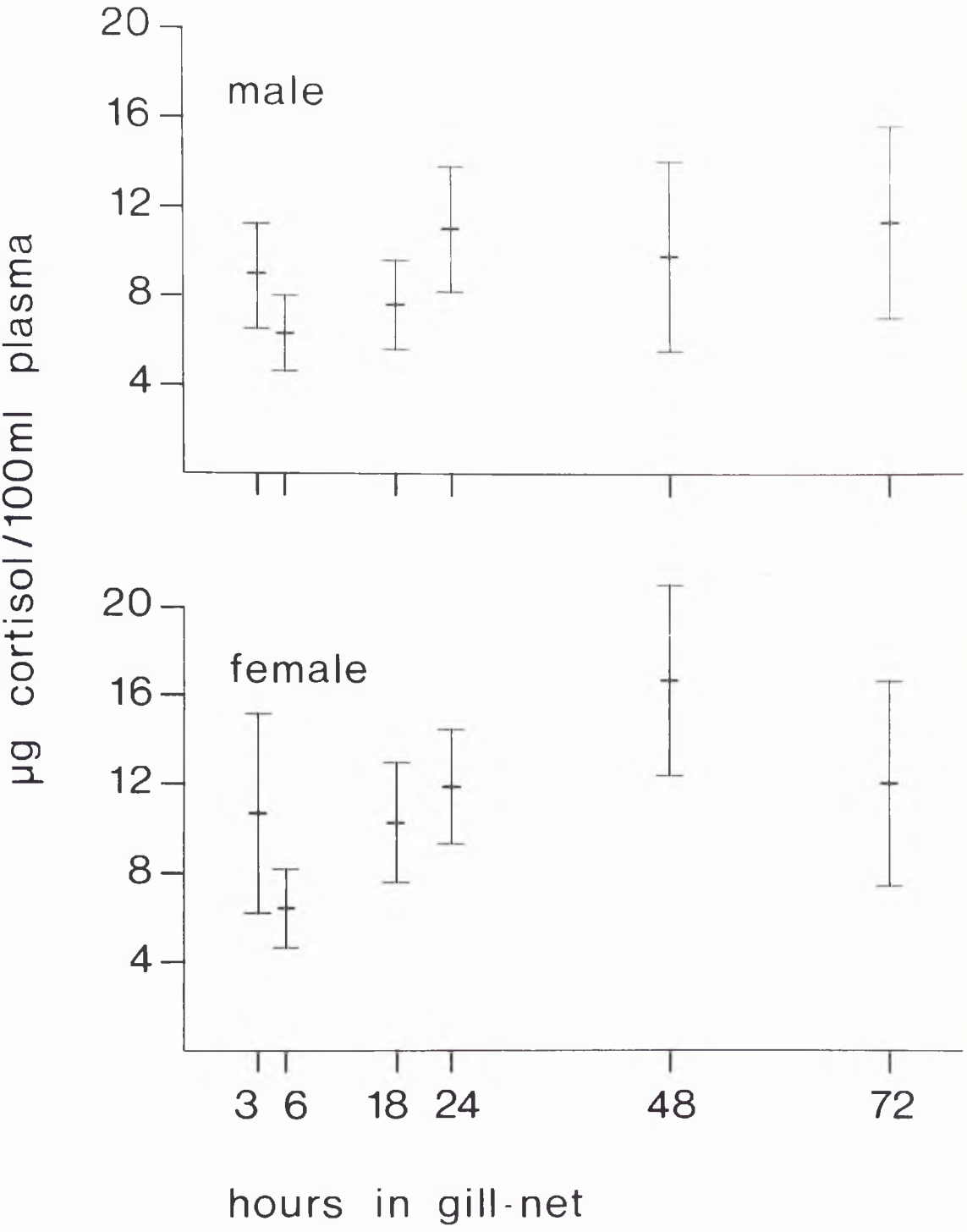


Fig.19. Effect on cortisol levels of time spent in gill-net (mean ±1 S.D.).

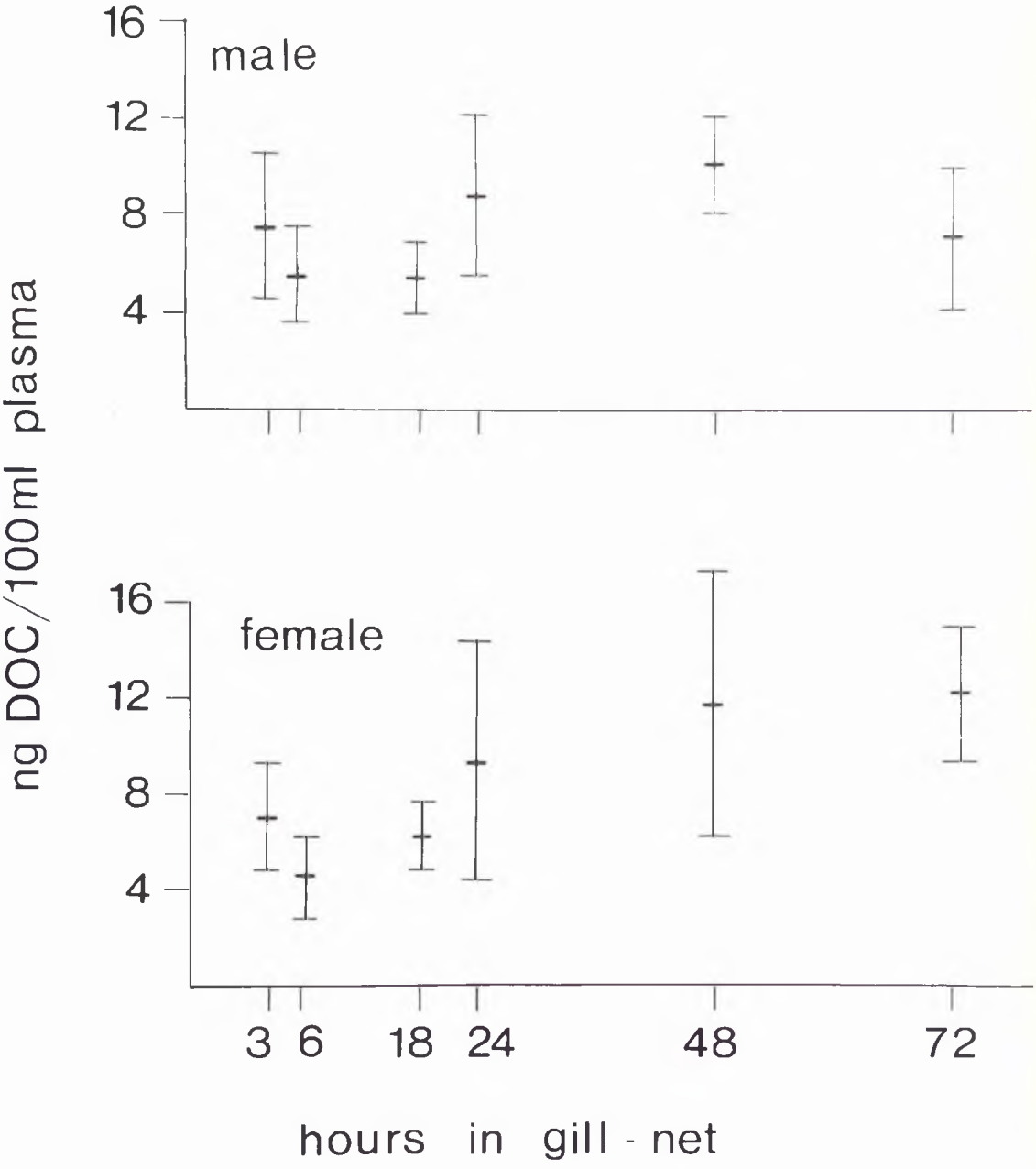


Fig.20. Effect on DOC levels of time spent in gill-net (mean ±1S.D.).

(a) Cortisol (Fig. 21). The number of individual plasma samples collected for cortisol analysis are listed in Table 11.

No significant difference was found in cortisol levels throughout most of the year (comparing levels in February, May/June and December, p values > 0.05). However, in both males and females gill-netted in September, cortisol levels showed a significant rise compared to previous levels in May ($p < 0.01$ and $p < 0.001$ for males and females respectively). The levels in males rose from 8.8 ± 3.8 (S.D.) to 15.4 ± 7.2 (S.D.) and in females from 12.6 ± 3.4 to 20.5 ± 6.0 (all levels in µg/100 ml.). Seine-netted fish in September showed a corresponding rise in the males from 2.0 ± 1.3 to 5.2 ± 2.0 ($p < 0.001$) but the small rise in the females from 3.1 to 3.7 was statistically insignificant ($p > 0.05$).

The other significant changes in cortisol levels were found in females caught at spawning time in January. During this time females were caught at different stages of ovulation (p. 15), both on and off the spawning grounds. A progressive rise was found in cortisol levels in the three ovulatory stages but highest levels were found in females which were caught on the spawning ground. Pre-ovulating females showed a significant rise ($p < 0.01$) in cortisol levels, from 7.2 ± 3.3 (S.D.) to 12.8 ± 3.4 (S.D.), caught on and off the spawning ground respectively. Likewise, barely ovulating females caught off the spawning ground had levels of 7.6 ± 2.8 (S.D.) but were 13.3 ± 2.7 (S.D.) in fish caught on the spawning ground ($p < 0.001$). The greatest rise in cortisol levels was in ovulating females caught on the spawning ground, the mean level was 24.7 ± 7.1 (S.D.) compared to levels of 10.3 ± 2.7 (S.D.) in ovulating fish caught in open water off the spawning ground ($p < 0.001$). The sixteen ovulating females caught on the spawning ground all had cortisol levels exceeding those found in females caught at all other times of the year.

Atretic oocyte resorption. During the course of this study, 6 females were caught whose ovaries were undergoing atretic resorption, one in February (presumably having failed to ovulate the ripe eggs at spawning time), four in May and one in September. There was no significant difference in plasma cortisol levels of these females compared to non-atretic, mature females, caught at the same time of year by the same netting technique (Table 12a).

Fungal infection. Specimens were also collected heavily infected with Saprolegnia. These individuals have cortisol levels comparable to non-infected fish, at the same stage of maturity, caught in the same month by the same netting technique (Table, 12b). However, for seasonal sampling these infected individuals were not included.

(b) DOC (Fig. 22). The number of pooled plasma samples collected for DOC measurements are listed in Table 11.

Male DOC levels did not vary significantly during the year (p values > 0.05), the mean value was 6 ng/100 ml. and the range 2 - 9 ng/100 ml. Levels in females were comparable to levels in the males and again showed no significant variation in mean values in gill-netted fish. Insufficient plasma was available from seine-netting to allow statistical comparisions in these seasonal samples. At spawning time the mean DOC levels in barely ovulating and ovulating fish were slightly higher than at any other time during the year but not at a statistically significant level ($p > 0.05$).

TABLE 11: Fish taken for seasonal cortisol and DOC measurements.

Month, 1975	Cortisol (No. of samples from individual fish)	DOC (No. of pooled samples, each from 5 - 10 fish)	Reproductive state	net
January	45	6	pre-ovulating ♀	Gill
	12	3	barely-ovulating ♀	"
	16	-	ovulating ♀ (off spawning ground)	"
	16	7	ovulating ♀ (on spawning ground)	"
	52	12	ripe ♂	"
February	10	10	spent ♀	"
	10	10	spent ♂	"
May/June	10	7	mature ♀	"
	10	11	mature ♂	"
"	10	2	mature ♀	Seine
"	10	5	mature ♂	Seine
August/ September	10	6	mature ♀	Gill
	10	6	mature ♂	"
"	10	5	mature ♀	Seine
"	10	5	mature ♂	"
December	10	5	mature ♀	Gill
	10	5	mature ♂	"

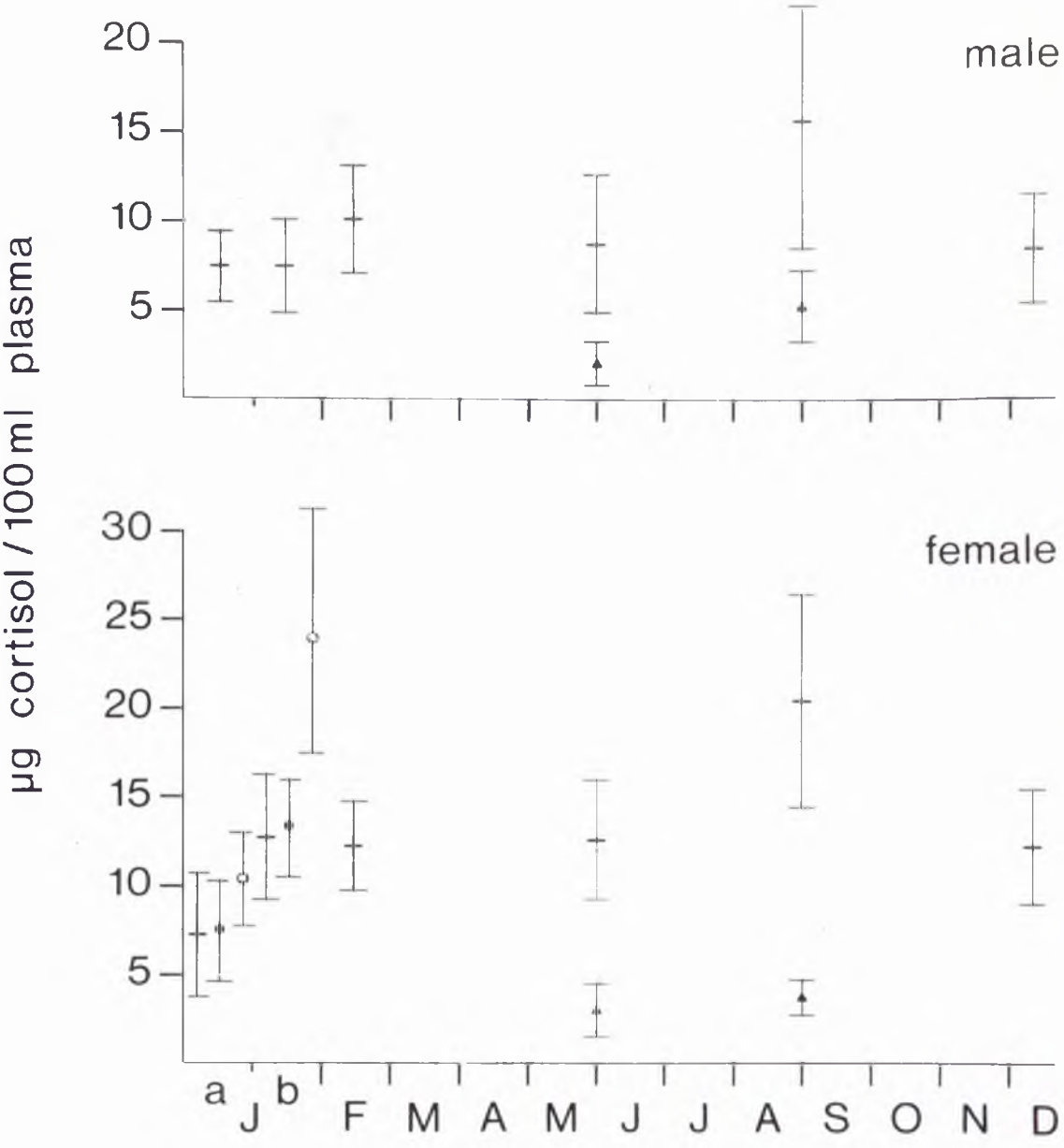


Fig. 21. Seasonal cortisol levels (mean ±1S.D.) a – off spawning ground, b – on spawning ground.

▲ – seine-netted, — – gill-netted.

● – barely ovulating, ○ – ovulating.

TABLE 12a: Cortisol levels in females with atretic ovaries.

Month	Net	Non-atretic comparative cortisol level µg/100 ml.	Cortisol in atretic fish µg/100 ml.
February	Gill	12.2	11.9
May	Seine	3.1	2.5
May	Gill	12.6	13.2 9.9 15.8
September	Gill	20.5	21.3

TABLE 12b: Cortisol levels in powan heavily infected with *Saprolegnia*.

Month	Net	Non-infected comparative cortisol level µg/100 ml.	Cortisol in infected fish µg/100 ml.
September	Gill	15.4	16.2 14.6 14.0 12.2
	Seine	5.1	3.9 4.9

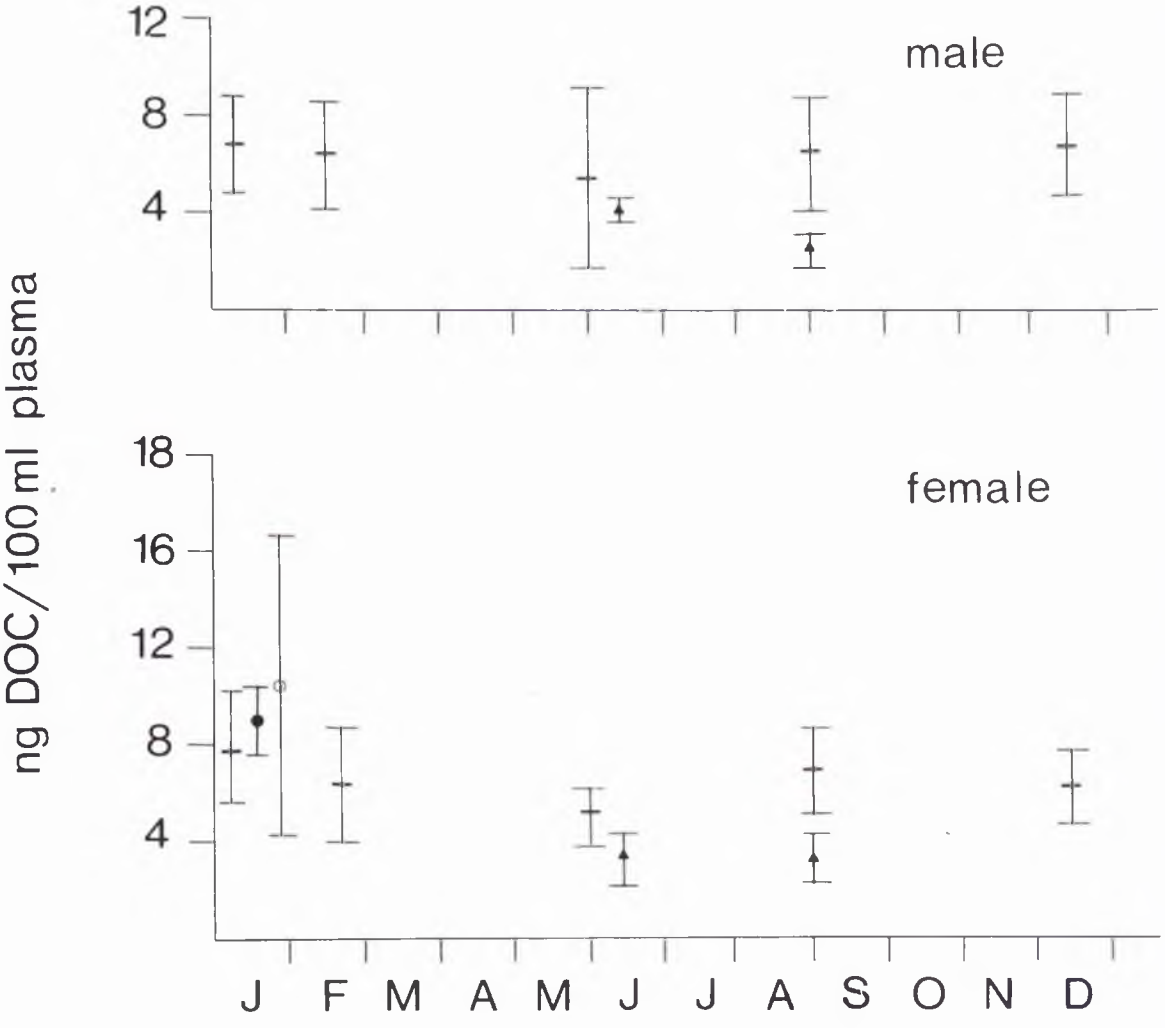


Fig. 22. Seasonal DOC levels (mean ±1S.D.).

C. DISCUSSION.

From experiments 1 - 4, on catching and handling techniques, employed, it is clear that the ideal way to make seasonal comparisons of DOC and cortisol levels in the powan would be to seine-net, and to kill the fish with MS222, throughout the year. Unfortunately this was not practicable. The problems of seine-netting have been discussed (p. 12) and in this case particular difficulty was experienced when winter gales precluded seine-netting on the spawning grounds in two successive years. As MS222 did not lower steroid levels in gill-netted fish these specimens were killed by concussion. Elevated levels in gill-netted fish are not due to the length of time spent in the net, (experiment 4), and fish kept in aquaria had high cortisol levels, even after 3 days. These facts indicate that restraint may be a stressing factor causing elevated steroid levels, as powan were held in reduced water levels preventing normal swimming behaviour in aquarium maintenance, and restraint when fish become trapped in gill-nets may produce similar stress effects, though less extreme. These results are in accordance with previous work on the salmon species (Fagerlund, 1967). Anaesthesia of powan with MS222 provides the lowest cortisol levels, perhaps by minimising the period of restraint, though levels were unaffected in gill-netted fish which were presumably already stressed by restraint in the net. It was found that immediate bleeding of gill-netted specimens produced lower steroid levels than keeping fish in aquaria for 1 - 3 days. It is possible that these levels would return to basal levels if fish were kept longer in aquaria, but it was not possible to maintain fish for longer periods. Maintenance was only possible using field-station facilities where suitable aerated tanks were not available, transport to the laboratories to more sophisticated aquarium facilities was over a distance of 80 miles, powan are highly sensitive to such

treatment despite mild anaesthesia, and were dead on arrival or within 24 hrs. thereafter. Therefore, only short-term maintenance was achieved. Again, it is postulated that the species' intolerance to restraint in captivity results in difficulty in keeping powan in aquaria and in high mortality even after a few days.

The investigation of bleeding fish immediately after catching was stressed since in previous steroid studies the investigators have not also been able to obtain specimens directly, themselves, and the time lag before bleeding the fish is clearly significant in the difficult conditions of obtaining fish from their natural environment.

Elevations in seasonal cortisol levels. Although gill-netting causes elevation of cortisol levels, this catching technique had, of necessity, to be used to obtain seasonal samples over the annual reproductive cycle. Consequently, seasonal levels (fig. 21) were certainly higher than in nature, and small variations occurring naturally may be masked by the stress response. However, major seasonal variations were apparent showing significant differences despite the overall high values.

The present work shows an elevation of cortisol levels, seemingly progressive in pre-ovulating, barely-ovulating and ovulating fish caught on the spawning grounds. This rise in cortisol level may be a transitory response to some event, not necessarily ovulation. It is possibly a stress effect (Selye, 1946) to which ovulating females may be sensitive because of their physiological state, though not necessarily directly concerned with the ovary. However, if on the other hand, the cortisol rises are not linked with spawning events, it is surprising that ovulating females caught off the spawning grounds do not show such rises. Osmoregulation and senescence have been suggested as being involved in cortisol

rises at spawning (p. 75) but these factors do not apply to the powan studied which are non-migratory and do not die after spawning. Scott (1963) described a similar peak of adrenocortical activity at spawning in the non-migratory species, Phoxinus phoxinus (histological evidence). Scott also implies increased adrenocortical activity in atretic females, though in this study on the powan, atretic females did not have elevated cortisol levels.

The autumnal rise in cortisol levels is also difficult to account for at present, but a number of possibilities exist:

(i) The high cortisol levels coincide with the resumption of gonad maturation in both sexes and also with the September rise of the somatic condition factor (fig. 5).

(ii) The rise could be a response to ecological influences associated with the rise of epilimnetic water temperature during the summer months (Slack, 1957) or the change from mainly planktonic feeding to benthic feeding at this time (Slack, Gervens and Hamilton, 1957).

(iii) The rise could reflect migratory patterns of the ancestral anadromous population (Maitland, 1970).

(iv) The rise could reflect intraspecific social interaction (Greenberg, 1974) though nothing is known at present of the ethology of the powan in this respect.

DOC levels. It was not possible to confirm the findings of Goswami and Sundararaj (1971a, b), from their in vitro work on Heteropneustes fossilis, that DOC is elevated at spawning time, eliciting maturation and ovulation of oocytes. It would be of value to find whether or not plasma DOC levels

in the same species rise at spawning time, however, DOC has not yet been identified in the plasma of Hataxanneustes fossilis.

Though DOC levels in male and female powan were consistent throughout most of the year, there was considerable variation in DOC levels in females at spawning time (fig. 22). The highest DOC levels identified were in ovulating females (reaching 18 ng), although other ovulating females had levels of only 4 ng. It is possible that DOC does rise at a very precise time at spawning but that the high level does not persist for any length of time such that it becomes masked in pooled samples by accompanying low DOC levels from other ovulating females. Measurement of a greater number of females taken at spawning time might clarify this. There is also the complication that the females caught in January were caught off the spawning ground. In view of the differences in cortisol levels in fish caught off the spawning ground from those caught on the spawning ground it is possible that an even greater rise in DOC levels might have been found in the latter group had they been available.

It is of interest to note that the DOC levels in the powan were found to be in the same range as levels in normal human plasma (2.8 - 16 ng/100 ml.), since it is an advantage that techniques already established for routine clinical analysis on human plasma can more easily be modified for use on teleost plasma if the same limits of sensitivity and range are required. The only previous DOC estimation in teleosts (p.34) by Chavin and Singley (1972) found 800 ng/100 ml. serum. The present results are in contrast with this very high level found in the analysis of an isolated sample. It is also noticable that, whereas in mammals DOC levels rise alongside plasma cortisol elevations, and proportionally DOC rises are greater than accompanying cortisol rises, in the powan rises in DOC levels were on a

much lower scale than mammalian elevations (Wilson, 1973). It is possible that secretory rates and accumulation of DOC in the plasma of teleosts do not occur to the same extent found in mammals.

It is clear from the cortisol and DOC estimations in the powan, that considerable individual variation occurs in males and females. Therefore, in any similar studies on teleosts particularly on individual plasma samples, measurement of sufficient numbers of samples is necessary in order to distinguish between individual variation and significant changes in steroid levels.

CHAPTER 6.

THE PITUITARY-ADRENOCORTICAL-OVARIAN AXIS

A. INTRODUCTION

Although there are embryogenic differences in the origin of the pituitary gland between teleosts and those of other vertebrate classes (Gorbman and Bern, 1962) and consequently the adult anatomy and histology of the teleost pituitary are characteristically distinct, their homologies are now well understood (Ball and Baker, 1969; Holmes and Ball, 1974). It is evident that, apart from the evolutionary differences in detail, the pituitary glands in all the vertebrate classes have similar cell types, the same general functions, and similar relationships exist between the pituitary gland and peripheral endocrine glands and target organs.

1. Pituitary-adrenocortical axis.

The pituitary-adrenocortical axis in mammals is well established. Evidence of similar feedback mechanisms between the teleost pituitary and adrenocortical tissue has been provided from results of physical and chemical extirpation techniques such as hypophysectomy and adrenalectomy and from administering exogenous adrenocorticotropic hormone (ACTH) and corticosteroids. These methods have been employed to study the effects on the histology of the pituitary and adrenocortical tissue, and, in fewer cases, to identify changes in steroid production by these tissues *in vitro* (review, Chester Jones *et al.*, 1969).

Though teleost ACTH differs in amino-acid sequence from mammalian ACTH (Scott, A.P., 1974), the basic 24 amino-acid unit is identical. Mammalian ACTH prevents the adrenocortical regression which occurs in hypophysectomised teleosts (Chavin, 1956; Pickford and Ats, 1957), and injection of mammalian

ACTH into intact fish causes hypertrophy of the adrenocortical cells. Injection of adrenocortical steroids evokes regressive changes in the adrenocortical cells, resulting in reduced cell size and nuclear diameter which indicates a negative feedback system (Hanke and Chester Jones, 1966; Oliveresu, 1966). Cortisol added to goldfish pituitary cultures inhibits ACTH release (Purrot and Sage, 1967). Similarly, dexamethasone (ACTH blocker in mammals) injected into rainbow trout reduces plasma cortisol levels (Fagerlund, McBride and Donaldson, 1968).

There are several reports of activation of the teleost pituitary-adrenocortical axis by stress or by ACTH administration (Rasquin, 1951; Chavin, 1956; Fagerlund, 1967, Fuller, unpublished). These reports describe hyperplasia of the adrenocortical cells and their degeneration in extreme cases attributed to stress. Similar histological changes in the adrenocortical cells and pituitary cells have been associated with maturation and spawning in some species (Robertson and Wexler, 1959) and with osmoregulatory changes (Hanke and Chester Jones, 1966; Ball et al., 1970). McLeay (1973[a,b]) working on Oncorhynchus kisutch, the Coho salmon, describes hypertrophy and hyperplasia of adrenocortical cells following ACTH injection. Increase in ACTH dosage resulted in increased cell size and nuclear diameter. Conversely, atrophy of the pituitary ACTH cells occurred following injections of cortisol. Hence, the existence of both the positive feedback from pituitary to adrenocortical tissue and the negative feedback from adrenocortical tissue to pituitary were indicated.

Evidence for a different type of pituitary control of adrenocortical function has been provided by Sundararaj and Goswami (1969). These investigators have, by *in vitro* experiments, found that luteinising hormone (LH) produced by the pituitary is an indirect ovulating agent acting by

eliciting an increase in corticosteroid secretion by the adrenocortical tissue. However, results of *in vivo* experiments are required to confirm these propositions and further evidence is required to support the hypothesis of corticosteroid involvement in ovulation in teleosts.

2. Pituitary-ovarian axis.

Information on te[illegible]ost gonadotropins is conflicting, though it is now widely accepted that LH is the major gonadotropic hormone present (Sundararaj, pers. comm.). In fact, although light- and electron-microscopy have revealed 2 gonadotropic cell types in the pituitary, no conclusive information exists, physiological or biochemical, for the presence of follicle-stimulating hormone (FSH). It is probable that histological differences may reflect one cell type at different stages of activity. The reproductive hormones and their functions have been well reviewed (Dodd, 1960; Hoar, 1965). It is now widely recognised that the gonadotropins regulate both gametogenesis and steroid production in both sexes. In the males development of sperm from the spermatogonia stage appears to be under pituitary control as does vitellogenesis in the female. Secondary sexual characters are also controlled by the pituitary, though probably indirectly via the gonads.

Fish prolactin (LTH) is produced in the teleost pituitary and has also been associated with reproduction and parental care. This hormone is not identical to mammalian LTH and its functions in teleosts remain unclear though most evidence suggests its role is in osmoregulation (review, Ball, 1969). Sundararaj and Goswami (1965) have shown that prolactin stimulates growth and secretion of the testis in *Heteropneustes fossilis* though the physiological validity of their findings has not been confirmed.

3. Adrenocortical-ovarian axis.

Evidence for the adrenocortical-ovarian axis is less well established. Information has been provided mainly from work on one species, Heteropneustes fossilis, by Goswami and Sundararaj (1971a, b). Their findings have already been described (p. 80). In summary, they postulate that LH secretion elicits secretion of cortisol and DOC from adrenocortical tissue at spawning time and these corticosteroids are then responsible for maturation and ovulation of oocytes. In vivo experiments are lacking and the work has not been repeated in other species.

The above evidence is further confused by reports from Colombo et al. (1973). Three species of teleosts, Lentocatus armatus, Gillichthys mirabilis and Micropogon proximus were studied. Ovarian incubates of all species were able to produce DOC from progesterone-4-^{14}C as precursor. The authors suggest that their information supports the contention of Sundararaj and Goswami, that DOC may be involved in ovulation and maturation but the study of Colombo et al. rules out the possibility of the adrenocortical-ovarian axis if, as they suggest, DOC is produced by the ovary itself. Though it is surprising to find the enzyme systems for DOC synthesis from progesterone in ovarian tissue, in vitro production of DOC does not confirm that this also occurs in vivo.

Aim of this section of the project.

The aim of this section of the work was to discover any evidence for a pituitary-adrenocortical-ovarian axis in the powan. Evidence available on other teleosts is at present conflicting, often unconfirmed and derives from experiments carried out on distantly-related species.

The reproductive cycle of the gonads, and the adrenocortical tissue and its steroids, have been investigated in previous sections of this work (Chapters 1, 2, 5). In the present chapter the anatomy and histology of the pituitary gland, and seasonal histological changes in the pituitary and adrenocortical tissue are investigated, also a series of *in vitro* experiments to determine the effect of corticosteroids on ripe ovaries was carried out.

B. MATERIALS AND METHODS.

1. Specimens.

Powan used in this section were obtained by the methods described in Chapter 1. The specimens were categorised as follows for comparative histological studies:

(a) Control fish were gill-netted in August, killed by concussion immediately after catching.

(b) Stressed fish were gill-netted in August, maintained in tubs of loch water for about 2 days. Individuals unlikely to survive longer were then killed by concussion.

(c) Ovulated fish were females gill-netted on the spawning grounds in January, killed immediately by concussion.

2. Pituitary histology.

Fixation, wax-embedding and sectioning of pituitaries was as described previously (p. 10).

A preliminary study was made of the powan pituitary gland. Serial sections, 4 - 5 µm, were made of 10 glands in sagittal section and 2 cut transversely.

Staining:

(a) General stain - Masson's Trichrome (Anon, 1969).

(b) Adenohypophyseal stains - Periodic acid-Schiff (Anon, 1969) modified, reagents - BDH magenta, technical grade was the only preparation of basic fuchsin to give consistently satisfactory results, it was used in conjunction with thionyl chloride. Lead-haematoxylin (MacConnail, 1947), stained for 3½ hrs, counterstained in 1% aq. erythrosin, 1 min.

(c) Neurohypophyseal stain - Paraldehyde fuchsin (Gabe, 1953).

3. Head-kidney.

Head-kidney histological preparations were made as described previously (p. 19).

4. Nuclear diameter comparisons.

The degree of stimulation of cell types was estimated by comparison of nuclear diameters in preference to cell sizes as the cytoplasm outline was often indistinct. A Watson shearing eyepiece was used to estimate nuclear diameters. Comparisons were made of results from control, stressed and ovulated fish. The cell-types studied were adrenocortical cells of the head-kidney and ACTH, pars intermedia and LH cells of the pituitary.

Nuclei diameters were measured in 30 cells, for each cell type, in each of the following mature individuals:

Adrenocortical cells	- 2 control females, 2 control males,
	- 2 stressed females, 2 stressed males,
	- 2 ovulated females.
ACTH cells	- 2 control females,
	- 4 stressed females,
	- 2 ovulated females.
LH cells	- 2 control females,
	- 2 stressed females,
	- 2 ovulated females.
Pars intermedia cells	- 2 control females.
	- 4 stressed females,
	- 2 ovulated females.

As few individuals were used in each case, statistical evaluation was carried out by one-way analysis of variance within all groups (assuming the maximum standard deviation) and applying the student t-test for small numbers of samples.

5. In vitro culture of ovaries.

All glassware was heat sterilised before use and transported in a sterile condition to the Loch Lomond field station. Culture medium was Wolf and Quimby Amphibian Culture Medium (Flow Laboratories, Ltd., Irvine, Scotland). Water, saline (7 g./l.) and a further amphibian medium provided by L. Lloyd, Zoology Department, St. Andrews University, (Priest, Lloyd, Callan, unpublished) were also used for comparison. Steroid hormones were

dissolved in propylene glycol as described by Goswami and Sundararaj (1971a, b). The culture procedure was carried out as described by these authors but omitting oxygenation of the medium during the culture period as all oocytes survived without this precaution. Pieces of ovary about 1 cm. cube were placed in 8 ml. of culture medium containing the various steroids (see below), culture was carried out at 20°C, for 20 hrs, with gentle shaking of the culture flasks throughout. The following steroids were used in attempts to induce maturation and ovulation of oocytes:

Steroid	µg/ml. medium
DOC	5, 10, 20, 40.
DOC acetate	5, 10, 20, 40.
Cortisol	5, 10, 20, 40.
Corticosterone	10, 20, 40.
Testosterone	10, 20, 40.
Estradiol	10, 20, 40.

Control flasks contained culture medium with an equivalent amount of propylene glycol added (without steroid) as was used for dissolving the steroids above. These experiments were performed on ovaries taken from ripe females collected by seine-netting in November, December and at spawning time in January.

C. RESULTS.

1. The pituitary.

(a) Anatomy. The adult powan pituitary is typically teleostean, with the adenohypophysis divided into 3 distinct regions (Plate 11a). The pituitary lies anterior to a saccus vasculosus and immediately posterior to the optic chiasma. The terminology of Olivereau (Ball and Baker, 1969) will be used for the adenohypophysis. The small rostral pars distalis lies anteriorly, the proximal pars distalis lies in the middle bulging out on both sides of the pituitary. The pars intermedia is atypical compared to most teleost pituitaries studied and represents an intermediate type between that found in the salmon, where the 3 lobes are almost dorso-ventrally arranged and the type of pituitary found in the eel where the 3 lobes are arranged antero-posteriorly (Holmes and Ball, 1974). In the powan, the pars intermedia lies beneath the proximal pars distalis but protrudes upwards into the latter and envelops the proximal lobe posteriorly. The pars intermedia is considerably reduced in size relative to the large proximal lobe in ripe and spawning specimens and at this stage lies only along the periphery of the proximal lobe. The neurohypophysis ramifies into the proximal pars distalis, to a lesser extent the rostral lobe, but mostly into the pars intermedia.

(b) Histology. Neurosecretory tracts run in the neurohypophysis to the pars intermedia which takes up neurosecretory stains strongly (Plate 12c). No stainable neurosecretion was found in the 2 pars distalis lobes.

(i) rostral pars distalis (Plate 22d, 12a). In the rostral pars distalis two main cell types were identified, typical of ACTH and prolactin cells found in other teleosts (Ball and Baker, 1969). The prolactin cells in

the pavan form follicles among which ramifies the neurohypophysis. The presumptive prolactin follicles are distinct in their appearance and are acidophilic in their staining properties. The cells stain with Orange G used in the Schiff's procedure and are negative to Schiff's stain. The ACTH cells are weakly acidophilic. These cells are characteristically elongated with elongated nuclei. The ACTH cells are more conspicuous in stressed specimens where their degranulated appearance also distinguishes them from other cell types.

(ii) proximal pars distalis (Plates 11b, c). Three cell types are identified in the proximal pars distalis; two are basophilic, the third is acidophilic.

Basophils: The two basophil cell types both take up general stain in the same way and are periodic acid Schiff-positive. However, the more dorsal cells stain intensely with paraldehyde fuchsin, these cells penetrate into the rostral lobe, this is typical of staining and location of thyrotropic cells in the pituitary. The more ventral basophil cells in the proximal lobe stain less intensely with paraldehyde fuchsin, though this method of distinguishing between the two basophil types was not always possible. However, the more ventral basophils increase dramatically in size in spawning fish and it is probable that these are the gonadotropic, LH cells.

Acidophils: The acidophils of the proximal lobe are negative to periodic acid-Schiff and paraldehyde fuchsin. The cells are small in comparison to the gonadotropic cells and run as collars of cells between the latter. The description is typical of somatotropic cells in teleosts (Ball and Baker, 1969).

(iii) pars intermedia (Plate 12b). Only one cell-type was identified in the pars intermedia. The cells are negative to periodic acid-Schiff but

stain with lead-haematoxylin. They are generally chromophobic in staining affinity. The pars intermedia is small at all stages of maturity particularly noticable with the disproportionate increase in size of the proximal pars distalis in spawning fish.

2. Comparison of cell types in normal, stressed and ovulated rohan.

Light microscopy did not reveal changes in appearance of adrenocortical cells in ovulating or stressed fish compared to controls. Although the adrenocortical tissue was abundant in these fish, this observation can also be accounted for by individual variation in the area of adrenocortical tissue which was observed in control fish. Likewise, using light microscopy it was not possible to show any significant difference in pars intermedia cells. However LH and ACTH cells showed some conspicuous changes in the different groups of fish. In LH cells of ovulating fish there was an increase in nuclear size, granulation of cytoplasm and the nucleolus was more distinct. In ACTH cells of stressed fish degranulation was conspicuous. The extent of these changes varied in different fish and ovulating fish showed no such changes in the ACTH cells.

As a more definite criterion for comparison, the nuclear diameters of the cell types were compared (fig. 25). The following increases in nuclear size were found to be significant:

(a) In adrenocortical cells of ovulated and stressed fish, compared to control specimens.

(b) In ACTH cells of ovulated and to a greater extent of stressed fish, compared to control specimens.

(c) In LH cells of ovulated fish compared to control specimens.

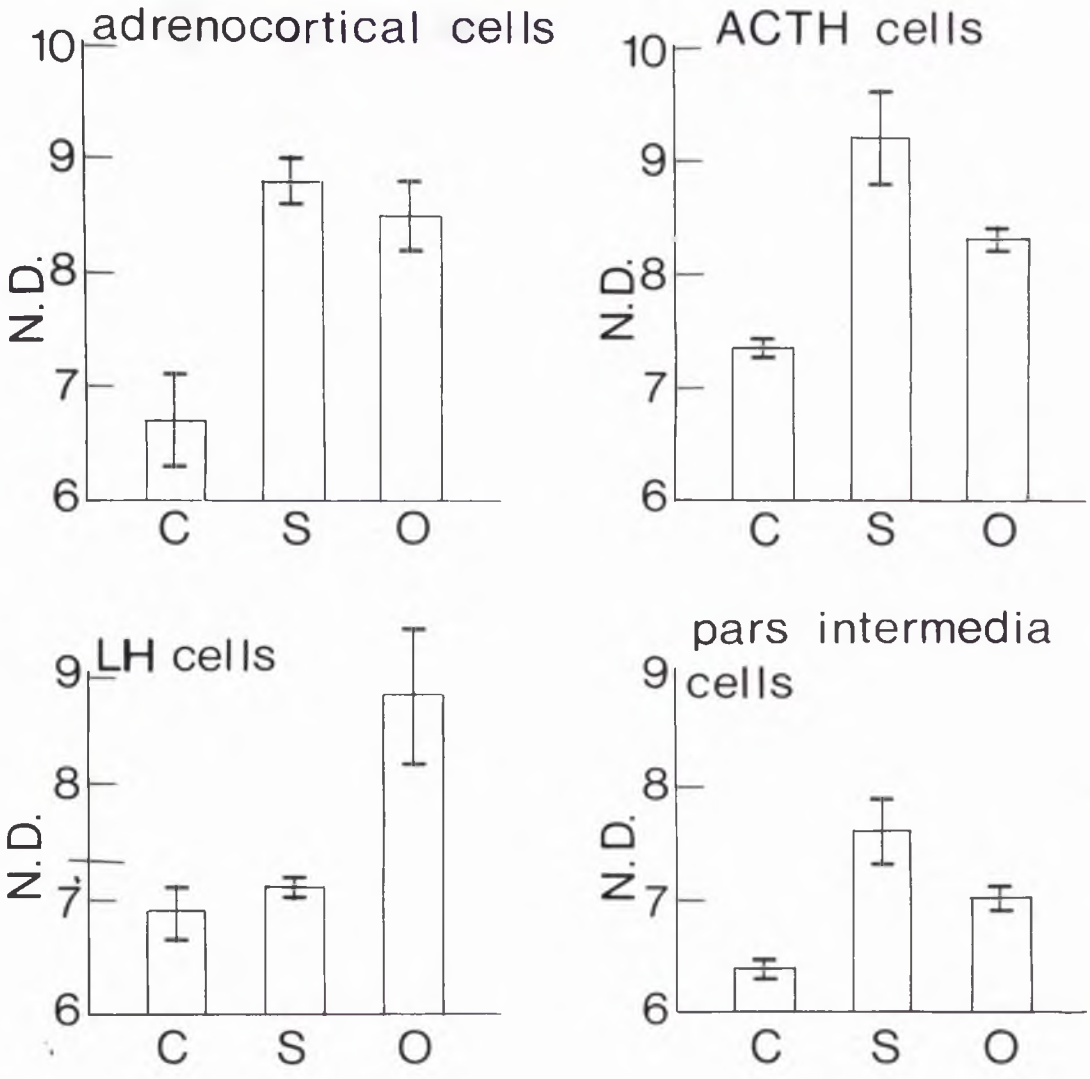


Fig.23. Nuclear diameter (N.D.) comparisons (mean ± 1S.D.), μm.

C-control, S-stressed and O-ovulated powan.

The appropriate p values are:-

		v Normal
adrenocortical cells	stressed	$p < 0.001$
	ovulating	$p < 0.001$
ACTH cells	stressed	$p < 0.001$
	ovulating	$p < 0.01$
LH cells	stressed	$p > 0.05$
	ovulating	$p < 0.01$
pars intermedia cells	stressed	$p > 0.05$
	ovulating	$p > 0.05$

Significance was accepted at the 1% level.

It seems, therefore, that LH and ACTH cells are stimulated in ovulating females. In stressed fish only the ACTH cells in the pituitary were stimulated. The ACTH cell changes in ovulating and stressed fish were accompanied by corresponding changes in the adrenocortical cells in the head-kidney.

3. In vitro culture of ovaries.

Both amphibian media proved satisfactory for culturing pawan ovary pieces for short periods of 15 - 24 hrs. The Wolf Quimby medium was chosen for the experimental procedures in order to reproduce the conditions used by Goswami and Sundararaj (1971a, b). Comparative cultures using water and saline as culture media resulted in early contamination and death of the oocytes. In spite of adhering to the conditions and precautions

described by Goswami and Sundararaj (1971a, b) no development or ovulation of the oocytes in Coregonus lavaretus was observed using the steroids listed above. The ovaries were examined whole and also for closer examination were fixed, wax embedded and sectioned, as described earlier (p. 10). No maturation or ovulation was induced by any of the steroids added to the culture medium, even at concentrations of 40 µg./ml. medium which was in excess of levels used by the above investigators who obtained 50% maturation and ovulation of oocytes using 5 - 10 µg./ml. medium of DOC and DOC acetate, cortisol had been similarly effective in their studies.

These experiments on powan ovaries collected one, two and three months before spawning-time all proved unsuccessful in repeating the findings of Sundararaj and Goswami on Heteropneustes fossilis.

D. DISCUSSION.

There is histological evidence for a change in pituitary LH and ACTH activity in Coregonus lavaretus at spawning time (pp. 109-10), and both histological and hormonal assay evidence for adrenocortical activity at the same time (pp. 109-10, 89-91). Stress, at any time of year, elicits pituitary ACTH activity and adrenocortical activity, recognised by the same criteria. There is, of course, gonadal activity (notably ovulation) at spawning time too. The causal relationships, if any, between these various physiological activities are, however, unclear.

The in vitro experiments (pp. 105-6) provide no evidence to corroborate the contention of Goswami and Sundararaj (1971a, b) that adrenocortical steroids (in particular DOC) elicit ovulation. This may be because of evolutionary differences in the two distantly-related species investigated - one a salmonid, the other an ostariophysan - or to different in vitro requirements of the two types of ovary; the large, yolky ova of Coregonus might be better maintained by a perfusion system.

LH activity, histologically established, is high at spawning, but there is no evidence to corroborate Goswami and Sundararaj's other contention that it is pituitary LH which stimulates the secretion of adrenocortical DOC. There is indeed a rise in adrenocortical activity at spawning time (though of cortisol, rather than DOC, pp. 89-91), but this could be accounted for by the simultaneous increase in pituitary ACTH activity.

It has been suggested on the basis of in vitro studies that, in the pituitary of Salmo gairdneri, the pars intermedia produces more ACTH than the pars distalis, and that during times of exceptionally high ACTH

secretion, the pars intermedia is the main source of ACTH (Scott and Baker, 1974). As pars intermedia cells in Coregonus lavaretus showed no significant change in nuclear diameter during either spawning or stress, this suggestion is not supported.

Thus although the present study adds to the evidence that adrenocortical activity is involved during spawning, in particular at ovulation, a causal relationship between adrenocortical, ovarian activity, and the role of the pituitary, are not yet confirmed.

GENERAL DISCUSSION

An essential preliminary to this investigation of the physiology of teleost adrenocortical tissue was the selection of a suitable species. Despite certain disadvantages, Coregonus lavaretus has been a satisfactory choice for this study. Phylogenetically, it is close to the basic stock of the main division of the teleosts, unlike the eel (Elopomorpha) and the herring (Clupeomorpha) which are in separate divisions so that the extensive studies already made of them can less reliably be extrapolated to other teleosts.

For a study of corticosteroid secretion rates, a species is needed in which the natural level of corticosteroids can be reliably assessed. Changes in steroid levels induced by catching, handling and aquarium maintenance techniques must be identified, and it is inadequate to work with fish "acclimatised" to aquarium conditions without thorough preliminary investigations as there is little information on how long acclimatisation takes. A period of 3 days has been used in the past for adaptation to captivity, but it is clearly not long enough in the powan as cortisol levels after 3 days were maximal, and similar elevations in cortisol levels of the salmon species, Oncorhynchus tshawytscha were found (Hane et al., 1966). Scott (1963) claims that symptoms of stress were discernible in minnows (Phoxinus phoxinus) even after six months in aquaria under optimal conditions. The ideal species must therefore be abundant throughout the year and catchable by a variety of techniques, particularly stress-free ones, preferably by the experimenter himself so that the techniques can be strictly monitored. This requirement rules out marine species. Powan satisfied these criteria, except in one respect, that the most stress-free catching method could not be used throughout the year. Seine-netting in Loch Lomond becomes impossible where the lake bed is littered with permanent

debris such as rocks, and shifting debris such as trees and wrecks, which shift during the winter gales. Clearing such areas is always difficult and often impossible. Such problems militated against seine-netting on the spawning grounds. The problem was aggravated by the fact that powan spawn in January, when the weather is a considerable factor to be reckoned with. Loch Lomond weather is often extreme - during January 1973 sampling, wind-speeds of 104 mph were recorded, the temperature is often below 0°C, and even in such a small area of water as Loch Lomond the funneling of wind and waves by hills and islands produces conditions which make fishing from a small dinghy hazardous, fishing however is always feasible. The short daylength at this season often entails fishing at night. All in all, a species spawning at a more wholesome time of year would be preferable.

The other disadvantage of the powan is the difficulty of maintaining specimens in aquaria for long periods. However, facilities for this are now being established in the form of a one metre deep, circular pond, five metres in diameter, with filtration equipment.

The size of the powan is favourable for this study; it is small enough to be easily manageable, yet large enough to provide an adequate sample of blood from individual fish, at least for cortisol estimations. The population is large, and statistically significant numbers of fish can generally be obtained.

Of British freshwater fish, the other salmonids, whilst allowing greater volumes of plasma to be extracted from individuals, have the disadvantage of osmoregulatory complications, except Thymallus and Salvelinus which are not available locally in any numbers. Cyprinids are near to their northern limit in Scotland, and only the minnow and roach are common in Loch Lomond. The minnow, although in all other respects ideal, is too small

for blood sampling. The roach is relatively common in Loch Lomond and has the advantage of spawning in the river Endrick (fig. 4) in May - June, but it is very much less abundant in Loch Lomond than the powan, and less background information on its biology there exists. The perch is common in Loch Lomond, and spawns conveniently in May, but its spawning sites are less easily delimited, its spawning season more diffuse, and the weedy nature of the waters it normally inhabits make seine-netting difficult. Worst of all, it is almost impossible to extract reasonable samples of plasma from perch. The european eel is abundant too, but belongs to an aberrant group of teleosts, and spawns in the Sargosso Sea, if it spawns at all (Tucker, 1959), after a complicated life-cycle involving two metamorphoses in the sea and in fresh water, and whose later stages may be senescent rather than pre-spawning. A more unsuitable species for this particular study is difficult to imagine. Predatory species such as pike are generally only available in small numbers.

Although the anatomy and histology of the pronephros have been described in over 200 species, many of the reports are surprisingly superficial. Little attention has been paid to details of the vascular supply and innervation, and in particular the chromaffin tissue is often glossed over. It is usually stated as an accepted fact that chromaffin tissue lies close to, or intermingled with, the adrenocortical tissue in the pronephros (e.g. Chester Jones, 1957). In other cases the location of the chromaffin tissue is either described without illustration, or shown only in diagram form, unconvincingly. Reliable histochemical identification of the chromaffin cells is virtually non-existent. In some species the tissue seems to be absent. In the present study both adrenocortical and chromaffin tissues have been definitively identified in the powan, and their relationships to the vascular and nervous supplies investigated more thoroughly

than in any previous study. It would be valuable to extend these studies, perhaps by peroxidase-enzyme immunostaining technique (Nakane and Pierce, 1967) to determine whether the various corticosteroids are secreted by different cells, or by the same cell; by improvement of the freeze-drying technique to identify more accurately the chromaffin cells; and by silver staining and electron-microscopy to provide more precise information on the innervation of the chromaffin cells.

It has proved possible, by GLC and by saturation analysis techniques, to show that all the major corticosteroids found in mammals are also present in the plasma of the powan, in the same order of magnitude as in mammals. This correspondence has the advantage that steroid estimation techniques routinely used for mammals can be applied directly to teleosts, with only detail modifications. However, the small volumes of plasma available from fish such as the powan necessitates modifications to this end. In the case of DOC, which is present in much lower concentrations than cortisol, other problems arise since pooling of plasma becomes necessary as modification of techniques used for mammalian plasma are difficult to apply to small plasma volumes due to limitations of sensitivity of the techniques. Hormones in even lower concentrations such as aldosterone may be even more problematic and for studies of these steroids it would be advantageous to choose a species from which greater volumes of plasma can be taken.

The source of the corticosteroids is presumably the adrenocortical tissue, but in view of the contention of Colombo et al. (1973), that DOC may be secreted by the ovary, it would be of value to compare corticosteroid levels before and after passing through the pronephros. In this study plasma was taken from the posterior cardinal veins leaving the pronephros.

It is not possible to say what the normal, resting level of cortisol or DOC is in the powan; the best approximation is the lowest level obtained, which occurred in seine-netted, MS222-killed fish. It is not unlikely that lower levels might be found in fish caught and killed by other methods. What is clear is that hormone measurements based on fish caught by unspecified methods, or by methods such as gill-netting, now shown to be stressful, or maintained in aquaria, are of dubious value. Where such techniques have of necessity to be used (as in gill-netting powan in January) comparative hormone levels from unstressed fish should be available. It was found in this study that, despite elevations of steroid levels in gill-netted fish, the range was remarkably constant (of cortisol and DOC) in both sexes, and statistically significant changes in the steroid levels could be identified outwith the elevation due to the catching technique.

The seasonal variations in corticosteroid levels are difficult to explain. There is a dramatic elevation of cortisol levels in ovulating fish on the spawning ground. This accords with the concept that corticosteroids are involved at spawning time and particularly ovulation (Scott, 1963; Goswami and Sundararaj, 1971a, b); but ovulating fish occur off the spawning ground with cortisol levels which, although higher than at other times of the year, are lower than the high levels in females on the spawning ground. Elevation in DOC levels in ovulating females off the spawning ground were not statistically significant. These facts suggest that whatever the cause of corticosteroid elevation on the spawning ground may be, it is not ovulation itself. Nor does it accord with Goswami and Sundararaj's contention that it is a rise in DOC, or the DOC:cortisol ratio (in favour of DOC) that elicits ovulation. Nor do the *in vitro* experiments on powan ovaries confirm the Goswami and Sundararaj hypothesis as no corticosteroids elicited ovulation in ripe powan ovaries *in vitro*. This may be a technical problem, due to the difference in ovarian histology in *Coregonus lavaretus* and *Heteropneustes fossilis*.

The rise in cortisol levels in September is more difficult to explain. It coincides with many ecological, physiological and behavioural changes, but there is also the possibility that it may reflect osmoregulatory requirements of the ancestral anadromous populations. The latter aspect could be studied by examining one of the anadromous populations which still exist in the Baltic.

Finally, the study of pituitary and adrenocortical cell-types showed a direct relationship of the corticosteroid elevations with changes in ACTH cells and adrenocortical cells. These results provide evidence that the corticosteroids measured in this study were in fact produced by the adrenocortical cells of the head-kidney under ACTH control from the pituitary.

SUMMARY

This is a study of the physiological activity of the adrenocortical tissue in the teleost Coregonus lavaretus (Linnaeus) and some related salmonids.

1. Biology of Coregonus lavaretus, the powan.

As a basis for the study of endocrine changes, the biology of Coregonus lavaretus, in Loch Lomond, Scotland, is studied over a period of three years with special reference to the reproductive cycle. The reproductive cycle is analysed in terms of gonadosomatic ratios and gonad histology. Spawning takes place in January, on shallow, offshore gravel banks, several of which have been identified. Gonad recrudescence occurs chiefly between September and December, and at the same time the somatic condition factor rises from its minimum in June/July to its maximum in September.

2. Anatomy and histology of the pronephros.

A study is made of the pronephros and associated organs in Coregonus lavaretus, with particular reference to its vascular supply and innervation. The adrenocortical tissue is histochemically identified; it lies around the right posterior cardinal vein and its main tributaries. The chromaffin tissue is sparser and more intimately associated with the posterior cardinal vein and the sympathetic innervation of the pronephros. It too is critically identified by histochemical techniques. Comparative studies of related salmonids are also made.

3. Gas-liquid chromatography identification of corticosteroids in Coregonus lavaretus and related salmonids.

Gas-liquid-chromatography is used to identify corticosteroid hormones in the plasma of Coregonus lavaretus and in related slamonids, Salmo trutta trutta, Salmo trutta fario and Salmo salar. In all these species the corticosteroids present include cortisol, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 18-hydroxydeoxycorticosterone and aldosterone, in the same order of magnitude as in Homo sapiens.

4. Saturation analysis techniques for measuring cortisol and 11-deoxycorticosterone (DOC) in Coregonus lavaretus.

Saturation analysis techniques are established to measure plasma cortisol (by competitive protein binding) and DOC (by radioimmunoassay) in Coregonus lavaretus. The techniques are validated for accuracy, precision, sensitivity, specificity and reproducibility.

5. Effects of catching and handling methods on cortisol and DOC levels in Coregonus lavaretus.

The effect of different catching and killing techniques on plasma cortisol and DOC levels in Coregonus lavaretus are assessed. Seine netting followed by killing in a lethal level of MS222 produces the lowest levels of both steroids; gill-netting produces higher levels but this is not dependent on the length of time spent in the net. Maintenance in aquaria for up to 3½ days results in high cortisol and DOC levels. Post-morten delay before blood-sampling results in reduced steroid levels especially after 1 - 2 hours.

6. Seasonal variations in plasma cortisol and DOC levels in Coregonus lavaretus.

Seasonal variation in plasma cortisol and DOC levels in Coregonus lavaretus are determined in samples collected from fish throughout the year. Cortisol levels rise to very high levels in ovulating females caught on the spawning ground in January. Ovulating females caught off the spawning ground show elevated cortisol levels, but much less so. It seems, therefore, unlikely that the increase is causally related to ovulation, but to some other phenomenon associated with spawning.

7. The pituitary-adrenocortical-ovarian axis in Coregonus lavaretus.

Histological changes (in nuclear diameters) of the adrenocortical cells are directly related to corticosteroid level. An anatomical and histological study is made of the pituitary gland of Coregonus lavaretus and changes of adrenocorticotropic, ACTH, cells occur with corresponding changes in adrenocortical cells and the corticosteroid levels. Luteinising hormone, LH, cell diameters increase as the gonads mature. The nuclear diameter of the pars intermedia cells do not change.

Ripe ovaries of Coregonus lavaretus have been cultured in vitro and treated with DOC, DOC acetate, cortisol, corticosterone, testosterone and oestradiol and there was no indication of oocyte maturation or ovulation following these treatments.

The significance of these results in the context of our present knowledge of teleost endocrinology is discussed, and suggestions for further research are made.

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PLATE 2.

T.S. powan testis, 5 µm, lead haematoxylin/erythrosin.

sg - spermatogonia

sc - spermatocytes

st - spermatids

s - spermatozoa

(Zeiss Planapo 25, Ilford Pan F).

2A. Testis of maturing male caught in September.
Scale line = 50 µm.

2B. Testis of ripe male caught in December.
Scale line - 50 µm.

2C. Testis of spent male caught in late January.
Scale line = 50 µm.

2D. Testis of spent male caught in late February.
Scale line = 50 µm.

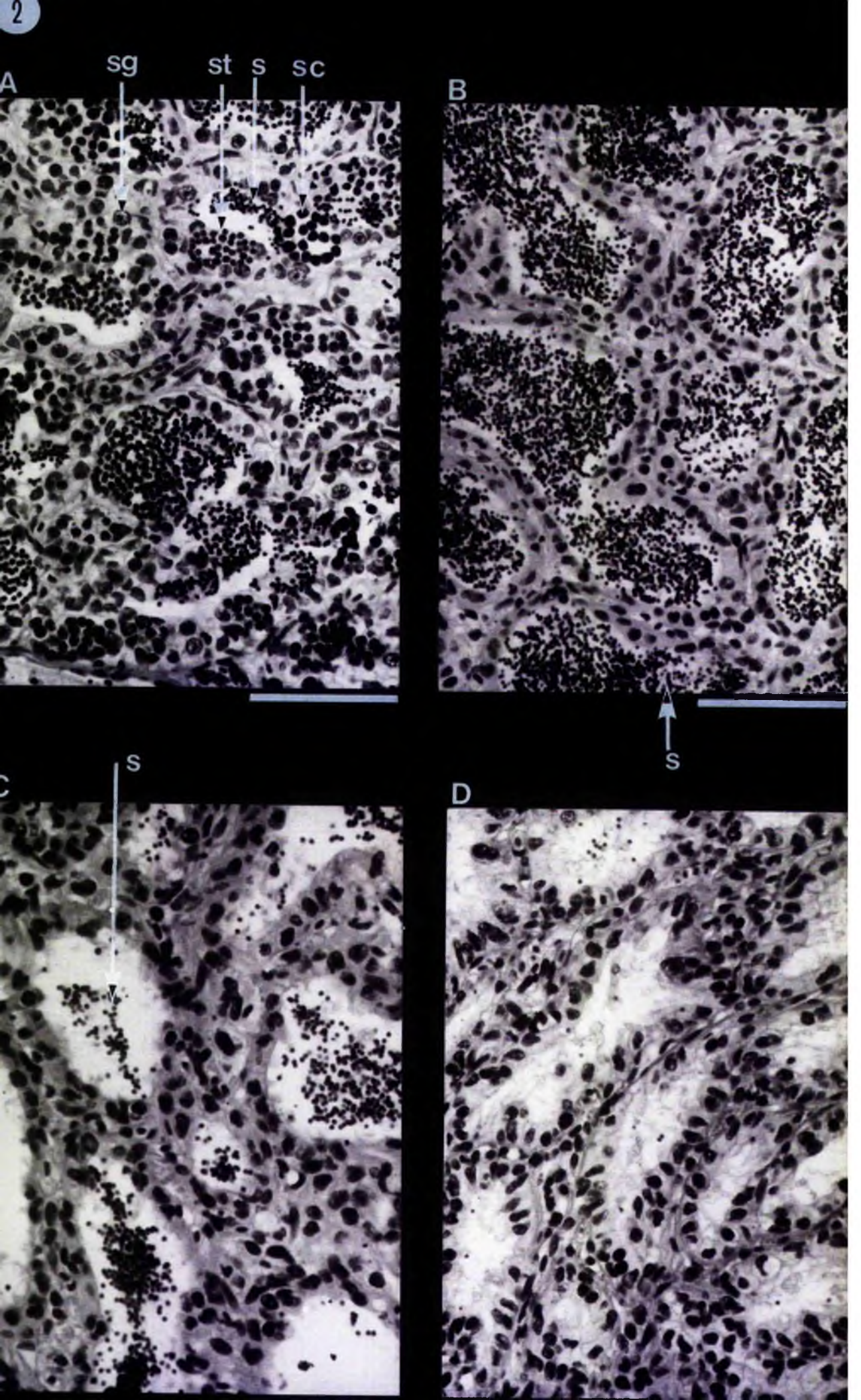

A
sg
st
s
sc
B
s
C
s
D

PLATE 3.

T.S. powan ovary, 5 µm, lead haematoxylin/erythrosin.

3A. Ovary of immature, year class 1 female. Primary oocytes showing nucleoli around periphery of nucleus.

f - follicle

Scale line = 350 µm.
(Zeiss Plan 16. Ilford Pan F).

3B. Ovary of mature female, May. Secondary oocytes showing deposition of yolk precursors.

f - follicle
o - coelemma
y - yolk precursors

Scale line = 350 µm.
(Zeiss Plan 16. Ilford Pan F).

3C. T.S. ovum of mature female, August.

o - coelemma
f - follicle
y - yolk

Scale line = 200 µm.
(Zeiss Planapo 25. Ilford Pan F).

3D. Ovary of ovulated female, February.

c - calyx of ovulated follicle
p - primary oocyte
a - atretic ovum

Scale line - 600 µm.
(Zeiss Planapo 10. Ilford Pan F).

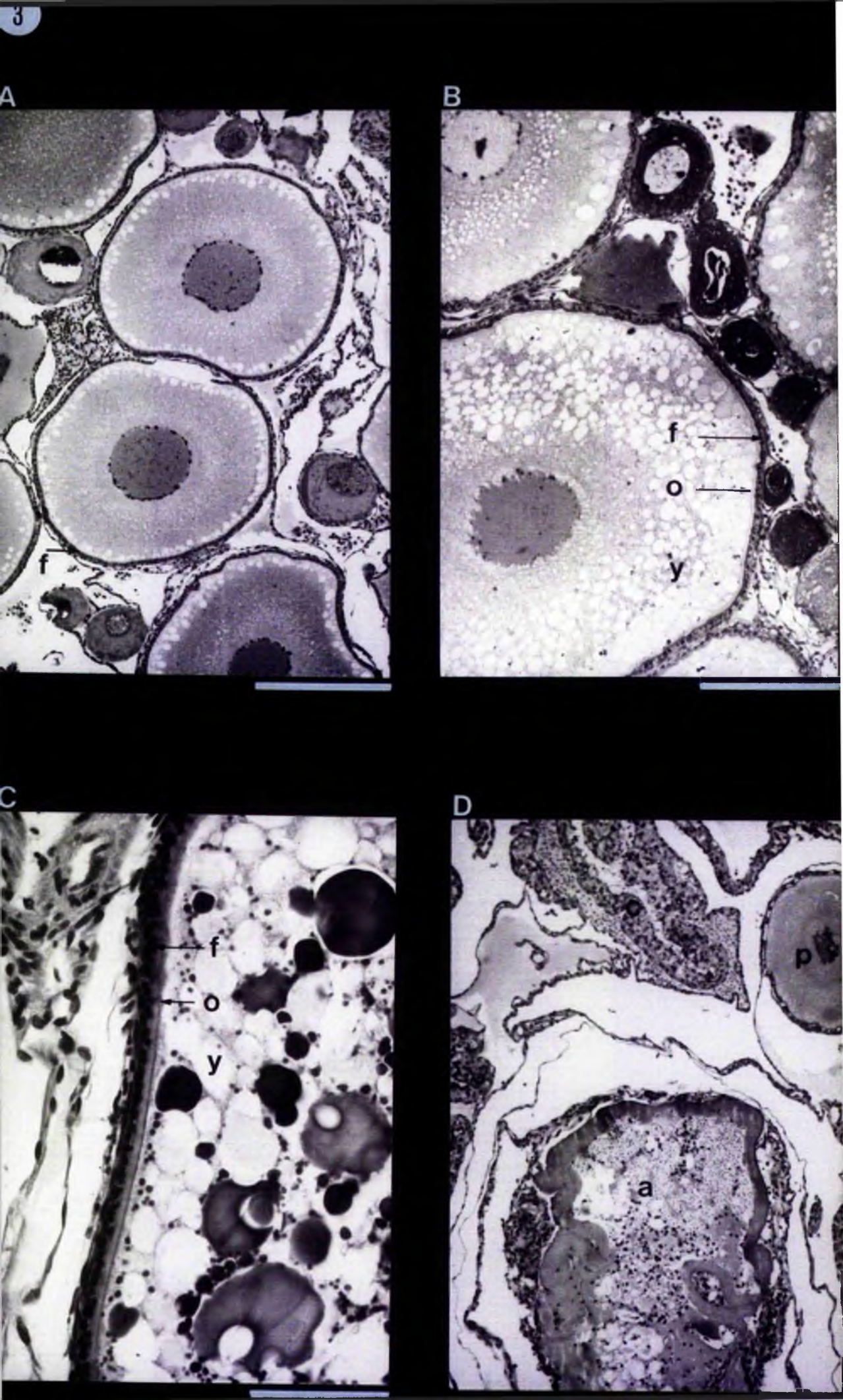

A
f
B
f
o
y
C
f
o
y
D
p
a

PLATE 4.

Dissection of powan, length 28 cm., to show location of pronephros.

c - digestive caecae
ov - ovary
d - right duct of cuvier
o - oesophagus
pcv - posterior cardinal veins
p - pronephros
m - mesonephros

(Makro-kilar 42 mm. Kodacolor II).

4A. Organs *in situ*.

4B. Gut, gonads, heart and peritoneum removed, showing right duct of cuvier from which blood was taken.

4C. Oesophagus removed.

4D. Specimen injected with white 'microfil' (Canton Bio-Medical Products Inc., Boulder, Colorado) to demonstrate vascularisation of meso- and pro-nephroi.

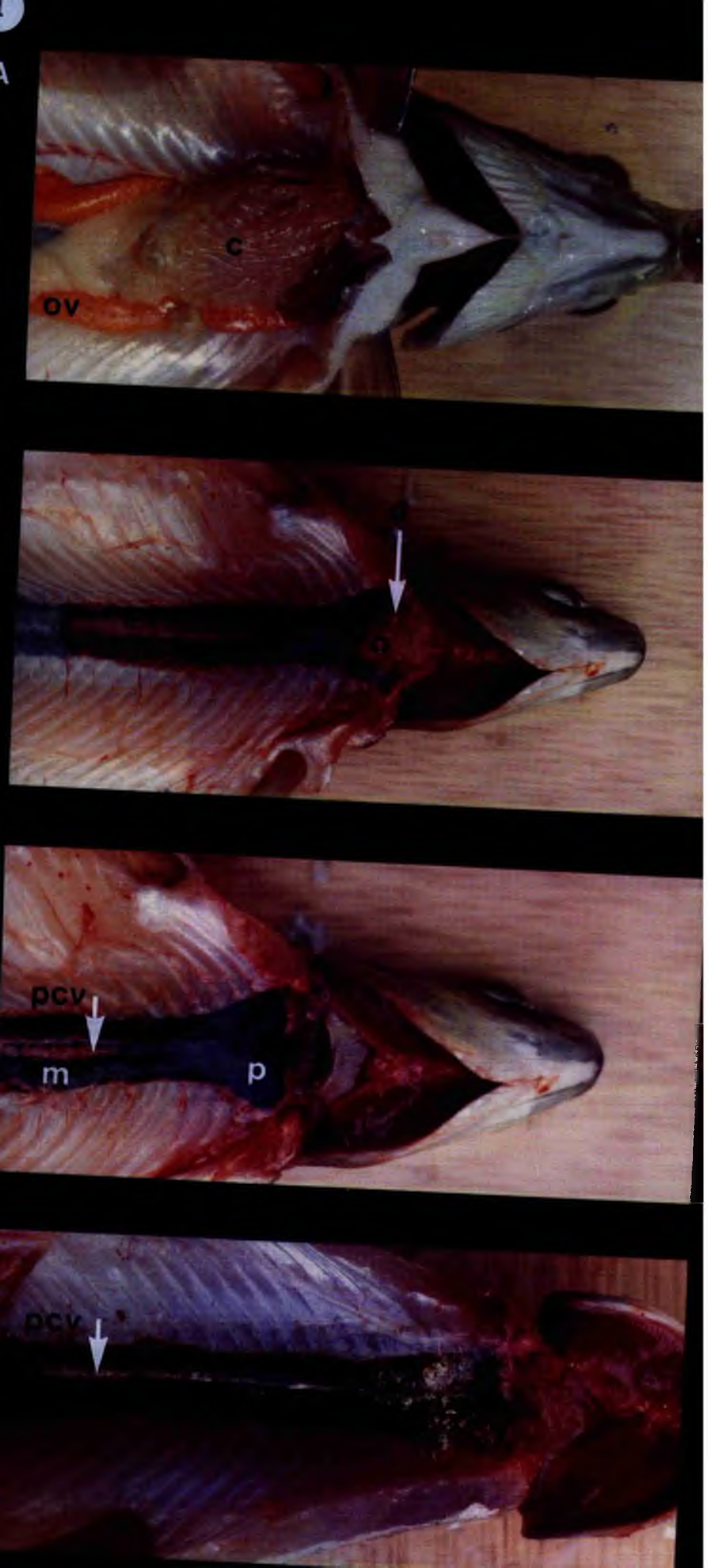

c
ov
pcv
m
p
pcv

PLATE 5.

Horizontal L.S. powan pronephros, 5 µm, Masson's trichrome.

a - adrenocortical cells
c - chromaffin cells
cm - coeliaco-mesenteric artery
l - lymphoid cells
m - melanophores
pcv - posterior cardinal vein
sg - sympathetic ganglion

5A. Scale line = 100 µm.
(Zeiss Plan 16. Ilford Pan F).

5B. Scale line = 35 µm.
(Zeiss Planapo 25. Ilford Pan F).

B

PLATE 6.

Cryostat, horizontal, L.S. powan pronephros, 10 µm.

a - adrenocortical cells
cm - coeliaco-mesenteric artery
l - lymphoid tissue
m - melanophores
pcv - posterior cardinal vein
sg - sympathetic ganglion

6A. Section stained by 3β-ol-dehydrogenase histochemical technique (pp. 20-22).

Scale line = 500 µm.

(Zeiss Planapo 10. Ilford Pan F).

6B. Adjacent section to 6A, stained in Masson's trichrome.

Scale line = 500 µm.

(Zeiss Planapo 10. Ilford Pan F).

6C. Section stained by 3β-ol-dehydrogenase histochemical technique (pp. 20-22).

Scale line = 300 µm.

(Zeiss Planapo 10. Kodacolor II).

6D. Adjacent section to 6C, stained in Masson's trichrome.

Scale line = 300 µm.

(Zeiss Planapo 10. Kodacolor II).

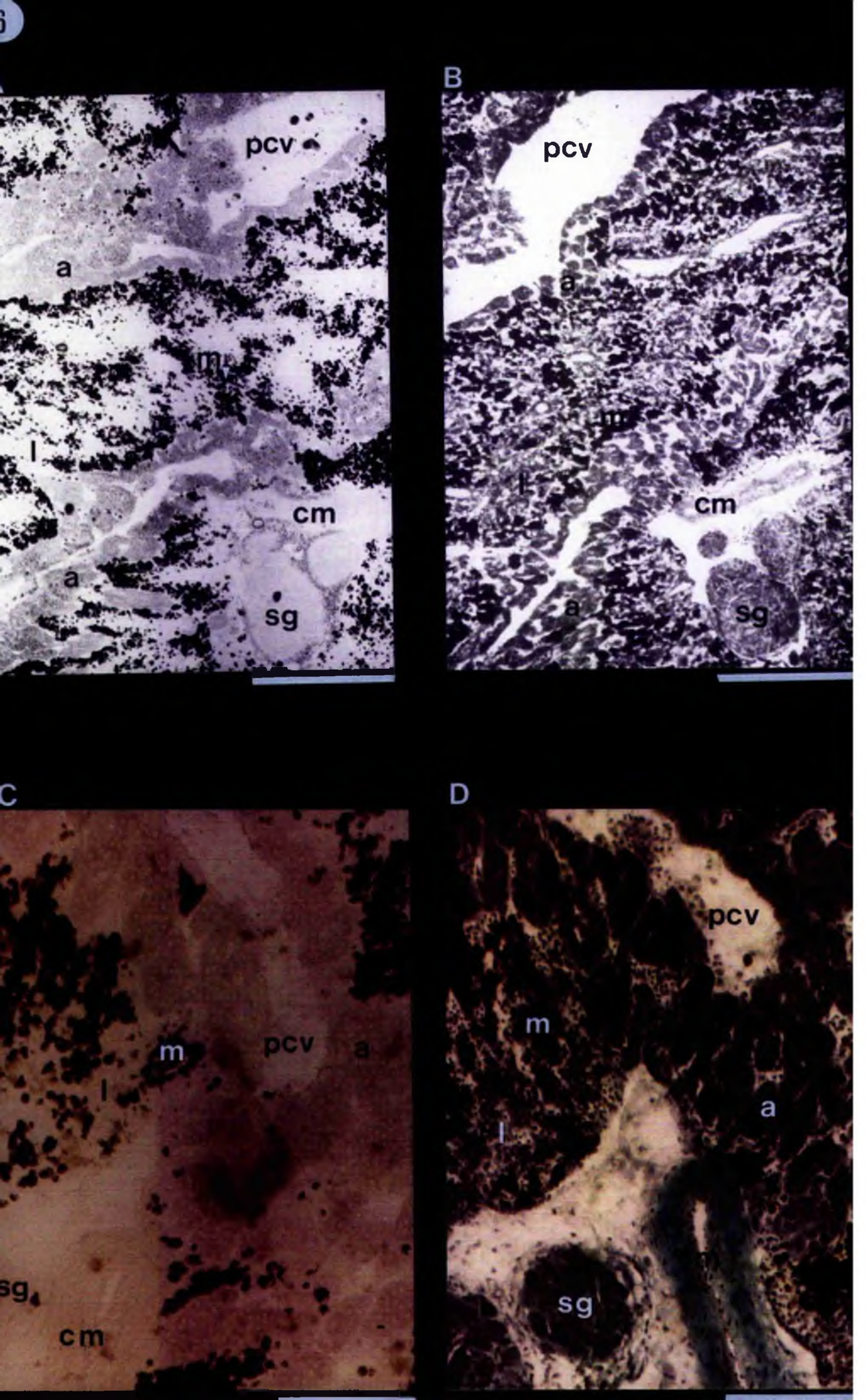

pcv
a
m
l
cm
a
sg
B
pcv
a
m
l
cm
a
sg
C
m
pcv
a
l
sg
cm
D
pcv
m
a
l
sg

PLATE 7.

Horizontal L.S. pavan pronephros, 10 µm, Masson's trichrome, for gross morphology.

a - adrenocortical cells
c - chromaffin cells
ca - coeliaco-mesenteric artery
n - nerves
pcv - right posterior cardinal vein

7A. Section at level E of Fig. 6.

Anterior to left.

Scale = 800 µm.

(Zeiss Plan 2.5, Ilford Pan F).

7B. Section at level E of Fig. 6.

Scale line = 250 µm.

(Zeiss Plan 6.3. Ilford Pan F).

7C. Section at level C of Fig. 6.

Scale line = 200 µm.

(Zeiss Planapo 10. Ilford Pan F).

7D. Section at level D of Fig. 6.

Scale line = 200 µm.

(Zeiss Planapo 10, Ilford Pan F).

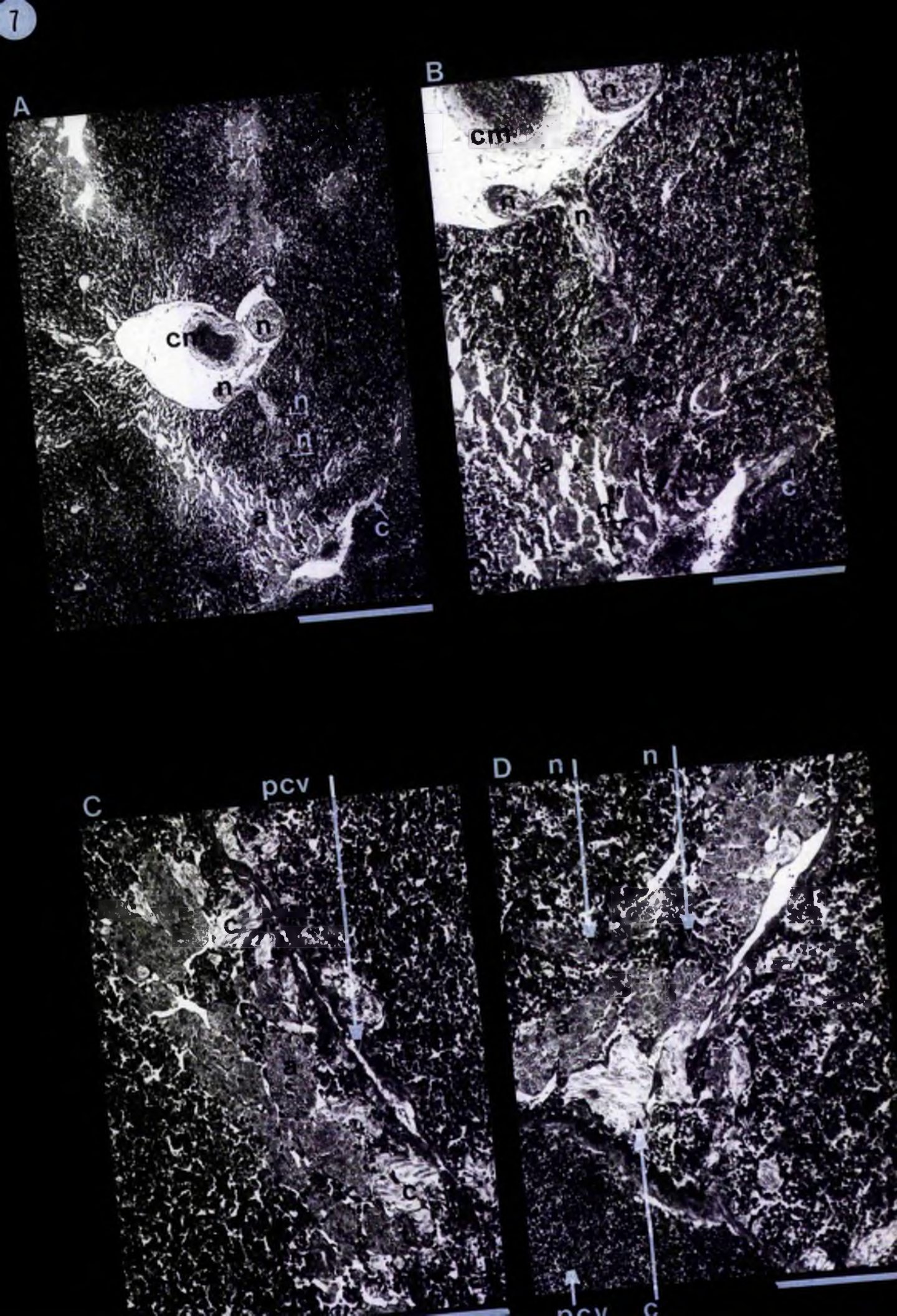

A
cm
n
n
n
n
a
c
B
cm
n
n
n
n
c
C
pcv
c
a
c
D
n
n
a
pcv
c

PLATE 8.

Cryostat T.S. pronephroi of powan (A, B) and rainbow trout (C, D), 10 µm.

a - adrenocortical cells
c - chromaffin cells
p - posterior cardinal vein

8A. Section stained in Masson's trichrome.

8B. Adjacent section to 8A, subjected to fluorescent histochemical technique (pp. 23-4).
Scale lines = 50 µm
(Neofluor 16. Ilford FP4).

8C. Rainbow trout pronephros, subjected to fluorescent histochemical technique (pp. 23-4).
Scale line = 50 µm.
(Neofluor 16. Ilford FP4).

8D. Sympathetic nerve ganglion fluorescing in pronephros of rainbow trout.
Scale line = 35 µm.
(Neofluor 40. Ilford FP4).

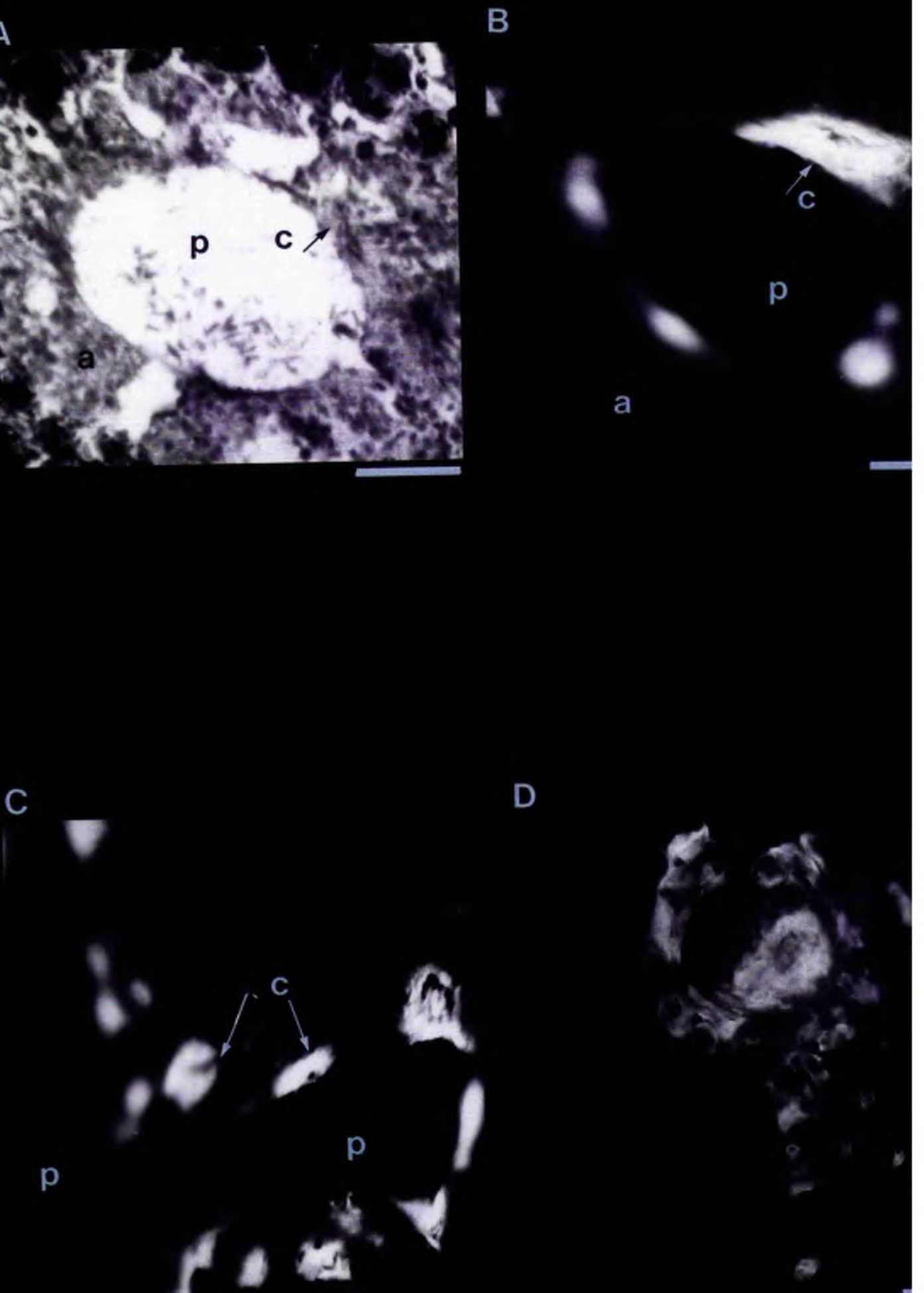

A
p
c
a
B
c
p
a
C
c
p
p
D

PLATE 9.

Sagittal sections of powan fry, 5 µm, Masson's trichrome.

a - adrenocortical cells
cm - coeliaco-mesenteric artery
da - dorsal aorta
g - glomerulus
l - lymphoid tissue
m - myotomal muscle
p - pronephros
pcv - posterior cardinal vein
pd - pronephric duct
sn - sympathetic nerve

9A. 6 month old powan.

Scale line = 100 µm

(Zeiss Planapo 10. Ilford Pan F).

9B. 3 month old powan.

Scale line = 100 µm

(Zeiss Plan 16. Ilford Pan F).

9C. 6 month old powan.

Scale line = 300 µm

(Zeiss Planapo 10. Ilford Pan F).

9D. 6 month old powan.

Scale line = 100 µm.

(Zeiss Planapo 25. Ilford Pan F).

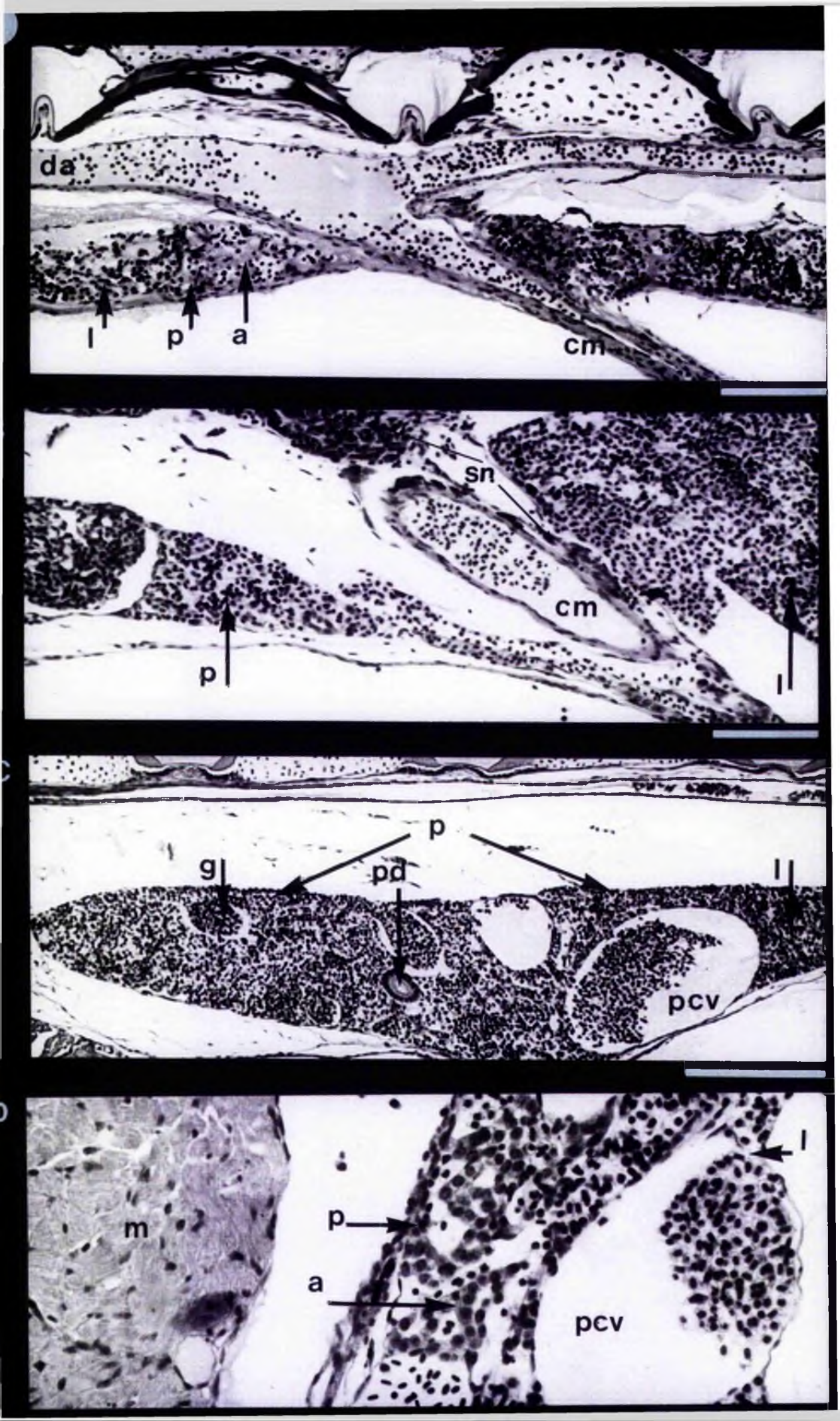

da
l
p
a
cm
sn
cm
p
l
p
g
pd
l
pcv
l
m
p
a
pcv

PLATE 10.

Horizontal L.S. of salmonid pronephroi, 5 µm, Masson's trichrome.

a - adrenocortical cells
c - chromaffin cells
l - lymphoid cells
m - melanophores
p - posterior cardinal vein

Sections from:-

10A. Salmo salar, the Atlantic salmon.

Scale line = 80 µm. (Zeiss Planapo 25. Ilford Pan F).

10B. Salmo trutta fario, the brown trout.

Scale line = 80 µm. (Zeiss Planapo 25. Ilford Pan F).

10C. Salvelinus willughbii, the char.

Scale line = 80 µm. (Zeiss Planapo 25. Ilford Pan F).

10D. Salmo trutta trutta, the sea trout.

Scale line = 80 µm. (Zeiss Planapo 25. Ilford Pan F).

10E. Osmerus eperlanus, the smelt. Two lobes of pronephros joined by bridge of lymphoid tissue.

Scale line = 500 µm. (Zeiss Plan 6.3. Ilford Pan F).

10F. Osmerus eperlanus, the smelt.

Scale line = 150 µm. (Zeiss Planapo 25. Ilford Pan F).

10G. Salmo trutta fario, the brown trout.

Scale line = 80 µm. (Zeiss Planapo 25. Ilford Pan F).

10H. Salmo salar, the Atlantic salmon.

Scale line = 80 µm. (Zeiss Planapo 25. Ilford Pan F).

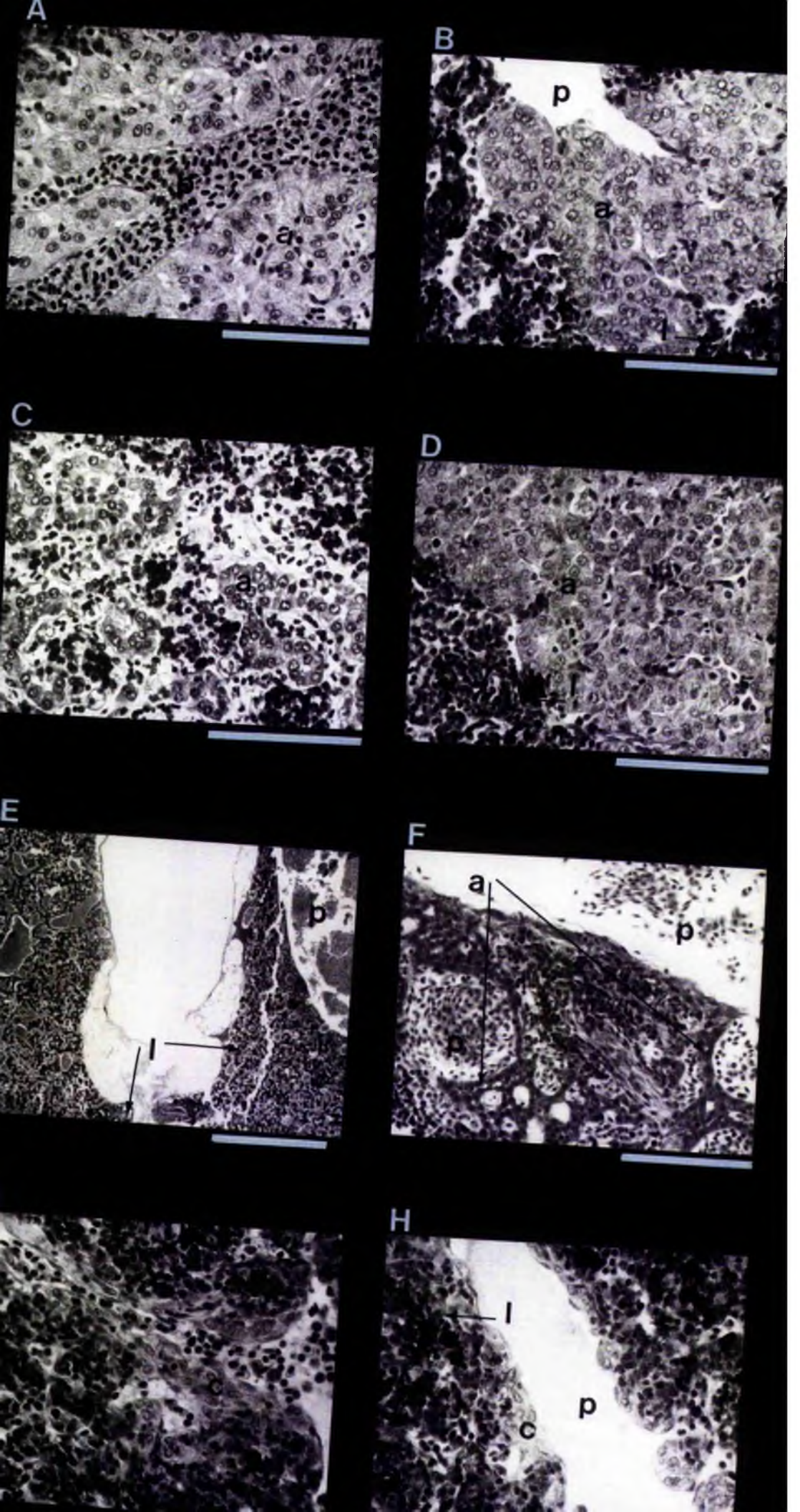

A
a
B
p
a
l
C
a
D
a
l
E
p
l
F
a
p
p
H
l
p
c

PLATE 11.

Median sagittal sections through adult powan pituitary, 5 µm, Masson's trichrome.

11A. Whole pituitary, anterior to left.

n - neurohypophysis
rpd - rostral pars distalis
ppd - proximal pars distalis
pi - pars intermedia

Scale line = 300 µm.

(Zeiss Plan 2.5. Ilford Pan F).

11B, B. Proximal pars distalis.

db - dorsal basophils
vb - ventral basophils
s - somatotropic cells
pl - prolactin follicle intruding from rostral pars distalis
n - neurohypophysis
v - blood vessel

Scale lines = 100 µm

(Zeiss Plan 16. Kodacolor II).

11D. Rostral pars distalis.

pl - prolactin follicles
n - neurohypophysis

Scale line = 50 µm.

(Zeiss Plan 16. Kodacolor II).

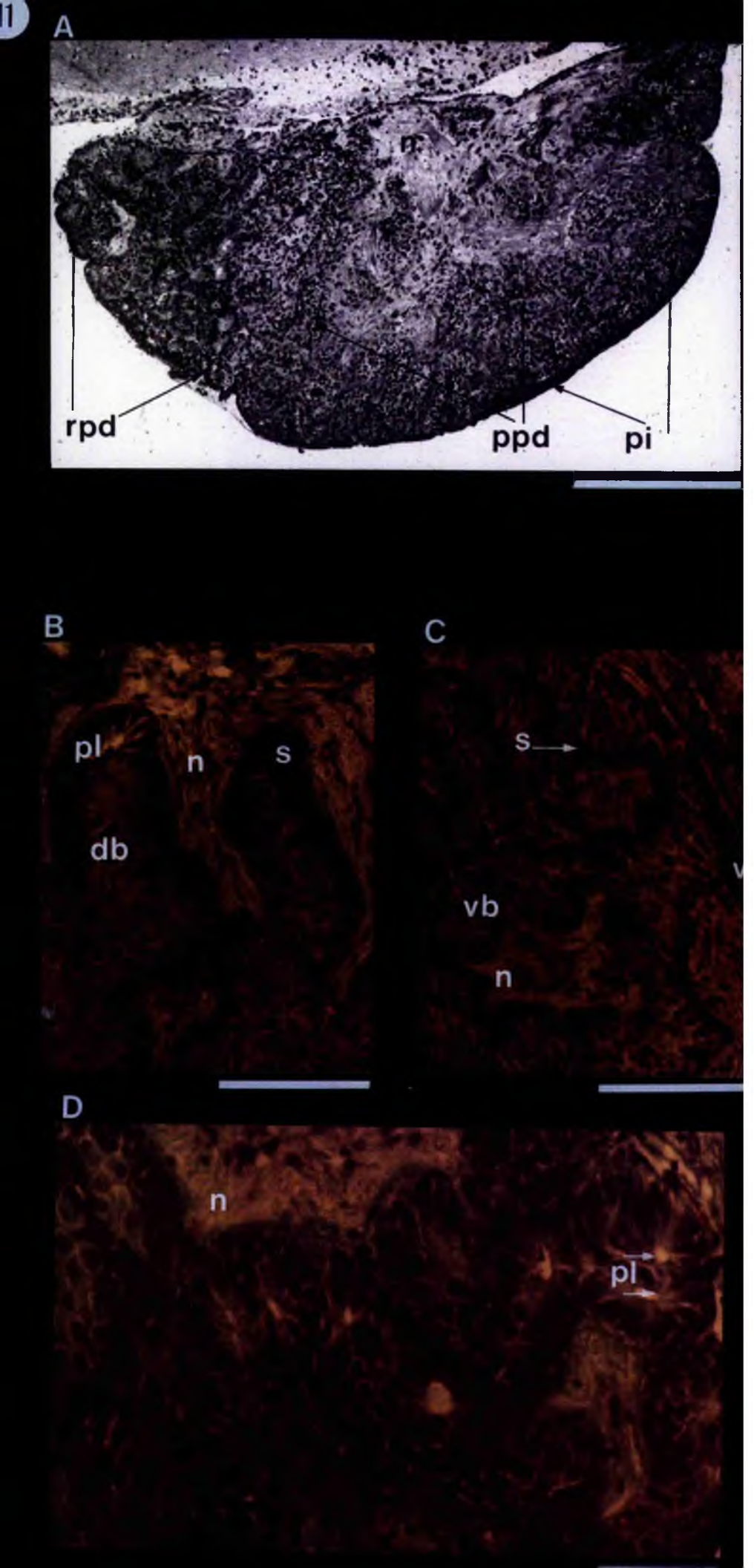

A
n
rpd
ppd
pi
B
pl
n
s
db
C
s
vb
n
D
n
pl

PLATE 12.

Median sagittal secyions through adult powan pituitary, 5 µm.

12A. Rostral pars distalis, Masson's trichrome.

a - adrenocorticotropic cells
n - neurohypophysis
pl - prolactin follicle
v - ventricle III of brain

Scale line = 100 µm.

(Zeiss Planapo 25. Kodacolor II).

12B. Proximal pars distalis and pars intermedia, Masson's trichrome.

b - basophils of proximal pars distalis
pi - pars intermedia cells
n - neurohypophysis

Scale line = 100 µm

(Zeiss Plan 16. Kodacolor II).

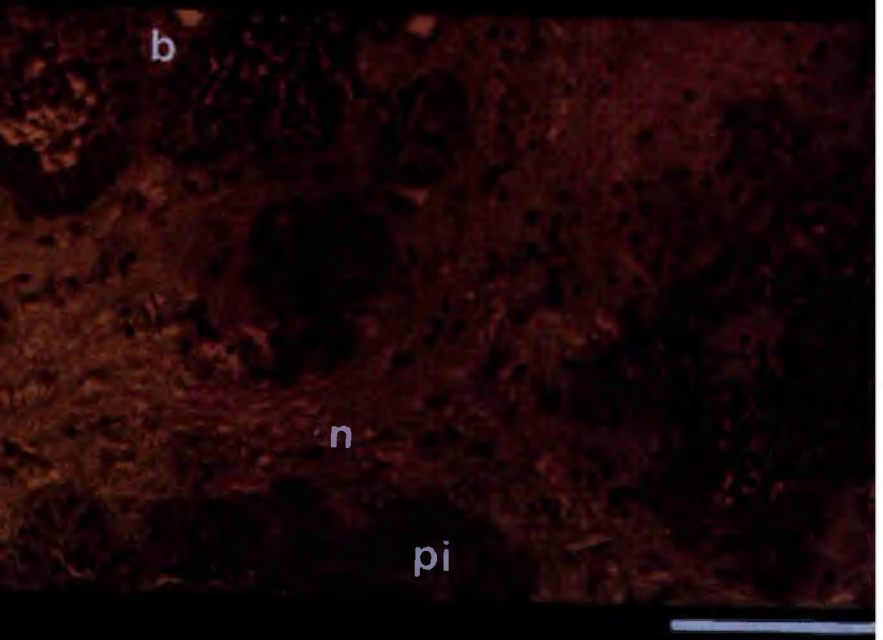
12C. Proximal pars distalis and pars intermedia, paraldehyde fuchsin, showing neurosecretory material.

b - basophils of proximal pars distalis
n - neurohypophysis.

Scale line = 100 µm.

(Zeiss Plan 16. Kodacolor II).


v
pl
n
a
a


b
n
pi


b
n